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GENERAL PHYSIOLOGY.

ANIMAL PATHOLOGY.

ETIOLOGY OF OROYA FEVER.

VI. Pathological Changes Observed in Animals Experimentally Infected with Bartonella bacilliformis.

The Distribution of the Parasites in the Tissues.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 11 to 14.

Experimental infection with *Bartonella bacilliformis* in young *Macacus rhesus* monkeys varies in severity according to the susceptibility of the individual animals[1,2,3] and is of three general types: (1) severe, fatal affection characterized by extreme anemia, similar to Oroya fever in man, (2) relative benign condition, in which there is moderate anemia or none at all, accompanied by localized skin lesions resembling those of human verruga, (3) mild systemic disease, involving the lymphatic glands and blood.

The pathological changes found in human organs and tissues after death from *Bartonella bacilliformis* infection have been described by Odriozola,[4] Arce,[5] Hercelles,[6] and by Strong and his coworkers.[7] The characteristic effects are seen in the liver, spleen, lymphatic glands, and bone marrow. Hypertrophy of the liver is almost invariable,[4,5] and the spleen is frequently enlarged,[4,7] though it may

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[1] Noguchi, H., and Battistini, T. S., *J. Exp: Med.*, 1926, xliii, 851.

[2] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

[3] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 715.

[4] Odriozola, E., La maladie de Carrion, Paris, 1896.

[5] Arce, J., *An. Facul. Med. Lima*, 1918, i, No. 1, p. 21; No. 2, p. 130; No. 3, p. 240; No. 4, p. 24.

[6] Hercelles, O., *An. Facul. Med. Lima*, 1918, i, 10.

[7] Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaburú, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

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be of normal size or less.[5]   Infarcts are commonly found in the spleen and some-
times also in the liver.[6, 7]   Strong and his coworkers note particularly the areas of
degeneration in the liver, beginning about the hepatic veins, apparently due to
the activity of the parasite and suggesting the presence of a toxin in the circulating
blood.   The lymphatic glands are enlarged everywhere[4, 5, 7] and frequently
edematous.   The hypertrophy is most marked in the mesenteric lymph nodes.[4]
The marrow of the long bones is red or mottled with red patches,[4, 7] and distinctly
softer than normal.   Microscopically there is evidence of phagocytosis by endo-
thelial leucocytes of red cells and polymorphonuclear leucocytes, also increased pro-
duction of normoblasts.[7]   Extensive phagocytosis by endothelial cells is found also
to occur in the spleen, while the lymphatic glands often contain many large swollen
endothelial cells in which the bacilliform parasites are present in large numbers.[7]
Similar intracellular parasites may be found[7] in the blood vessels near ulcerated
areas in the intestinal wall.   The parasite has a marked predilection for the
reticulo-endothelial system and sets up an active and persistent proliferation of
endothelial cells in the tissues or organs which it invades.

The characteristic nodular lesions caused by *Bartonella bacilliformis* have
occasionally been found to occur in the muscles and viscera as well as on the
mucous membranes.[4, 5, 8, 9]   Campodónico, in 1895,[10] reported the finding at
autopsy, in a young child who succumbed to severe anemia during the course of
verruga, of tiny red nodules in all the tissues of the body.

In the early experimental work with *Bartonella bacilliformis* only
the milder types of infection were observed, and it was not until the
third and fourth generations in monkeys that it was possible to in-
duce, by massive inoculations of passage strains, a fatal disease accom-
panied by extreme anemia comparable with Oroya fever of man.   Five
animals succumbed to the infection, and, as in the case of the human
disease, the conspicuous changes were found in the liver, spleen, bone
marrow, and lymphatic glands.   It was desirable, therefore, not only
to compare the findings with those occurring in human material, but
also to relate the histological changes in a particular tissue with the
presence or absence of the parasite.   In order better to accomplish
this purpose a number of infected animals were sacrificed either at
the height of disease, as evidenced by extensive local lesions and a
high titer of the parasite in the blood, or during the period of convales-

[8] Odriozola, E., *Crón. méd.*, Lima, 1914, xxxi, 157.

[9] Hercelles, O., *Crón. méd.*, Lima, 1914, xxxi, 67.

[10] Campodónico, E., *Crón. méd.*, Lima, 1895, xii, 43.

cence, when the blood findings were negative and the local lesions had subsided.

The important fact should be emphasized that tuberculosis was not encountered among the animals of the present series, notwithstanding special search was made for *B. tuberculosis* because of the possibility of confusion from this source.

Although microscopic examination of all materials—blood, organs, or tissues—was always made, the cultural method was found to be more reliable for determining the presence of *Bartonella bacilliformis*. Moreover, secondary infection is readily detected by this method. About 0.1 cc. of each of several dilutions (from 1:10 up to 1:1,000,000) of blood or saline suspension of tissue is introduced into leptospira medium, and the tubes are kept constantly at 25°C. for 4 to 5 weeks and examined periodically. Growth is often detected only after 2 to 3 weeks.

Films were always made of blood and impression smears of spleen, bone marrow, lymph glands, and liver. They were dried in air, fixed in methyl alcohol for 5 to 10 minutes, and stained with Giemsa's solution for 30 to 60 minutes.

Tissues were fixed in Regaud's fluid, and the sections stained with hematoxylin and eosin for histological study and with Giemsa's solution for detection of *Bartonella bacilliformis*.

The fourteen monkeys (*Macacus rhesus*) serving as material for the present study will be considered in three groups: (1) five animals which died of experimental infection with *Bartonella bacilliformis;* (2) five animals which showed signs of infection, locally and constitutionally, when sacrificed; (3) four animals which had shown signs of infection but were apparently in convalescence when sacrificed.

In the first group, three showed marked and two extreme anemia during the latter part of the disease or at the time of death. In Monkey 25 the red blood cells numbered 1,164,000 per c.mm. and the hemoglobin (Sahli) was 25 per cent; in Monkey 37 the red cells numbered 1,176,000 and the hemoglobin was 15 per cent. In these animals a terminal secondary bacterial infection intervened 12 and 96 hours before death.

There was moderate anemia in some of the animals of Group 2 but none in those of Group 3.

In Group 1 (fatal cases) the clinical picture was that of Oroya fever, and the pathological changes likewise proved to be similar to those

observed in the human infection. In Group 2 (non-fatal cases), the manifestations were characteristic of human verruga.

Pathological Findings in Fatal Infections with Bartonella bacilliformis.

The following outline is a composite picture of the characteristic changes noted. The findings in individual instances and the relation of the cultural to the pathological findings are recorded in Table I and in the protocols.

The duration of illness was 56 days in Monkey 17, 36 days in Monkey 25, 25 days in Monkey 30, 29 days in Monkey 35, and 41 days in Monkey 37.

External Appearances.

The skin and mucous membranes have a striking waxy yellowish appearance, and the numerous swollen lymphatic glands in the axillary and inguinal regions stand out prominently beneath the stretched skin. Pale nodules are found at the original sites of inoculation on eyebrows and abdominal skin. In an exceptional instance (Monkey 25) there were also numerous spontaneously occurring nodules on various parts of the body.

Thorax.

Lungs.—Pale yellow, normally crepitant, smooth. Congestion or edema at base in some instances. Bronchial lymph nodes greatly enlarged. Pleuræ anemic; small amount of effusion present.

Heart.—Myocardium flabby, pale, contains partially fluid pale red blood and occasionally a small clot. No petechiæ present. The pericardium contains some clear fluid. In one instance (Monkey 25) an acute pericarditis due to secondary invasion was present.

Abdomen.

Liver.—Pale, flabby in consistency, frequently somewhat enlarged, often shows yellowish mottling. Areas of necrosis and fatty degeneration are often recognizable.

Spleen.—Dark bluish red, enlarged, rather firm in consistency. Follicles indistinct. Scattered minute grayish red areas (infarctions) are present in all instances in varying numbers.

Kidneys.—Pale, with yellowish gray tint.

Adrenals.—Pale yellow.

Pancreas.—Normal in appearance.

Stomach.—Usually empty, mucosa pale.

Small Intestine.—Pale, serosa studded with numerous dark bluish swollen lymph nodes. Mucosa pale. Peyer's patches slightly hypertrophied.

Large Intestine.—Similar in appearance to small intestine, swollen lymph nodes on serosa giving it a striking aspect. No ulceration of the mucosa.

Bladder.—Normal in appearance.

Ovaries, Uterus, Testicles.—Pale, but normal.

Bone Marrow (Femur).—Dark grayish red.

Lymphatic Glands.—Entire lymphatic system hypertrophied and congested to an extraordinary degree. In the inguinal and axillary regions two or three swollen glands sometimes unite to form an irregular mass, 2 to 3 cm. in longest diameter, pink to deep red in color, and edematous. In the abdominal cavity the swollen lymph nodes are sometimes dark bluish, sometimes bluish red, and by contrast with the pale intestinal wall present a spectacular picture. They range in size from 2 to 6 mm. and are attached so firmly to the intestinal wall that they appear like verrucous nodules.

Nervous System.—The brain and spinal cord and their coverings are anemic but otherwise appear normal.

Microscopic Pathology.

Liver.—The normal pattern of cell arrangement is more or less disturbed. The hepatic cells have lost their sharp outline, stain poorly, and are vacuolated. The cells around the hepatic veins are necrotic (Fig. 1). Here and there, on the borders of the necrotic areas, between the liver cells, are engorged macrophages, which often contain erythrocytes, polymorphonuclear leucocytes, and erythroblasts. Around the portal veins are several layers of proliferated endothelial cells. In some specimens yellowish or brownish pigment in granules or small masses is found within the endothelial phagocytes or in endothelial cells lining the sinusoids. The degree of necrotic change around the hepatic veins is variable in the different animals. In some areas the liver cells are clear and homogeneous, in others coarsely granular. A few endothelial cells containing *Bartonella* were found after prolonged search in two of the five livers (those of Monkeys 17 and 30).

Spleen.—(Figs. 7, 8, 23.) Many infarctions are found, the periphery showing invasion of leucocytes. Many of the veins are occluded, owing probably to endothelial hyperplasia. The splenic nodules are reduced in size and are often scattered through the proliferating endothelial cells. Engorged macrophages containing erythrocytes are quite abundant. Yellowish brown pigment is present, some in the endothelial leucocytes, some between the splenic cells.

A number of endothelial cells containing masses of elements resembling *Bartonella bacilliformis* were found (Figs. 16, 25), but none of the swollen endothelial cells containing rods and granules such as were seen in human materials by Strong and his coworkers.

Lymphatic Glands.—The principal changes in these glands are the increase in the number of endothelial cells and the presence of numerous engorged macrophages. In the mesenteric nodes a large amount of greenish pigment is found in the phagocytic endothelial cells of the sinuses. *Bartonella bacilliformis* has been observed in a few endothelial cells in Monkeys 29 and 30 (Fig. 27).

Bone Marrow (*Femur*).—Numerous macrophages containing erythrocytes, polymorphonuclear leucocytes, and cell débris are present, and there are large numbers of normoblasts in some specimens (Monkeys 17, 25, 30). Some cells, probably endothelial, containing elements resembling *Bartonella bacilliformis* have been found in film preparations (Figs. 15, 26).

The changes are strikingly like those described as occurring in tissues from fatal cases of Oroya fever, the differences being in degree rather than in kind.   For example, the changes in the liver and spleen in monkeys are decidedly less pronounced than those seen in such human materials as are at my disposal for comparison, while the reaction in the lymphatic glands is in reverse degree, the more active phagocytosis being observed in monkeys.   The origin of the minute necrotic areas in the spleen may be sought in the obstruction of capillary lumina by proliferating endothelial cells.

As in the skin lesions, so also in the spleen and lymph glands, the progressive and persistent proliferation of endothelial cells is usually associated with the presence of *Bartonella bacilliformis* in considerable numbers, as detected by cultures.   The extensive zonal necrosis around the veins of the liver, which is less extensively invaded by the parasite, would appear, as suggested by Strong and his coworkers, to be due to the presence of a toxin in the circulating blood.

Pathological Findings in Animals Sacrificed during the Period of Active Infection.

The five animals of Group 2 all showed signs of active infection at the time when they were killed by etherization.   The period from inoculation to the time of killing was 22 days in Monkey 4, 68 days in Monkey 5, 53 days in Monkey 23, 24 days in Monkey 24, and 28 days in Monkey 29.   None of the animals showed severe anemia, although in Monkey 24 there was moderate diminution both in red blood cells and hemoglobin (erythrocytes 3,432,000, hemoglobin 55 per cent). *Bartonella bacilliformis* had been cultivated from the blood of Monkey 23 in a dilution of 1:100,000 16 days after inoculation, but it could not be recovered from undiluted blood 15 days later nor at any subsequent time.

The spleen and lymphatic glands were usually affected, and also the bone marrow as far as studied; the liver was less frequently in-

volved. Except in Monkey 23, of which special mention will be made later, the other organs were always normal in appearance.

The spleen was always more or less enlarged and rather firm; in one instance the surface was rather irregular (Monkey 5). In one animal (Monkey 23) there were numerous grayish brown areas of infarction, 1 to 1.5 mm. in diameter, scattered over the organ, which was a dark bluish red (Fig. 23). The lymphatic glands were in all cases swollen and congested and approached in size those of the fatally infected animals. The bone marrow was active in three animals studied (Monkeys 5, 23, and 24). The liver was affected to a similar degree in two animals (Monkeys 23 and 24); in the other three it was apparently normal. *Bartonella bacilliformis* was seen in a few of the endothelial cells in the liver of Monkey 24. The microscopical findings in the animals of Group 2 were identical with those in the fatally infected animals except that the changes were less pronounced in some respects.

An unusually interesting observation was made in the case of Monkey 23. On the pinkish surface of the lobules of the lungs were noticed a dozen pale, grayish, semitransparent, round nodules, measuring 1 to 2 mm. in diameter, firm, and sharply demarcated from the adjacent normal lung tissue (Fig. 22). Microscopically (Fig. 11), they consisted of numerous large mononuclear cells, many of them vacuolated and filled with dark granular pigment. The capillaries in this area, which were few in number, showed endothelial thickening. Several engorged macrophages and cell débris were also present. In a few of the endothelial cells *Bartonella bacilliformis* was found (Figs. 14, 24).

Pathological Changes in Animals Sacrificed during Convalescence.

Four animals were sacrificed when apparently in the course of convalescence from a mild infection. None showed fever or local lesions, though the lymphatic glands were still swollen in all instances. The erythrocyte count ranged from 5,472,000 to 6,584,000, and the hemoglobin was 80 to 90 per cent. The animals appeared to be in excellent condition when sacrificed 58 days (Monkey 7), 49 days (Monkey 8), 40 days (Monkey 10), and 30 days (Monkey 13) after inoculation.

The changes in these animals, when still evident, were characteristic. The spleen was somewhat enlarged in three instances and in two showed definite endothelial hyperplasia; in one (Monkey 13) there was a marked increase in fibrous tissue; in another (Monkey 7) the same changes were noted as had been found in the spleens of the actively infected animals. The liver was macroscopically normal in all four animals. The lymph nodes were active in two (Monkeys 7 and 8) but quiescent in the others. The bone marrow was normal in Monkeys 8, 10, and 13, and active in Monkey 7.

The findings suggested that Monkeys 7 and 8 were still infected and that Monkeys 10 and 13 had become free from infection. Cultural studies confirmed this inference, as will appear later.

DISTRIBUTION OF BARTONELLA BACILLIFORMIS IN THE TISSUES IN THE EXPERIMENTAL INFECTION.

In Oroya fever *Bartonella bacilliformis* persists in the blood until the patient's death. Whether or not various organs and tissues also contain the living parasite has not been determined. The pronounced changes found in certain organs have been assumed to be due either to invasion by the parasite or to the action of a toxin circulating in the blood. Cultural determination of the distribution of the organism in the tissues of experimental animals was, therefore, of special usefulness in establishing a relation between the pathological processes and the presence of the parasite. The results of cultural studies and of examination for pathological changes are recorded in parallel columns in Table I.

As the analysis shows, *Bartonella bacilliformis* was recovered from blood, liver, spleen, lymphatic glands, bone marrow, and local lesions in all instances of fatal infection, except when cultures were lost through contamination. The parasite was not so generally distributed in animals sacrificed during the course of an apparently non-fatal infection, though the local lesions and the changes in the spleen were rather severe. In Monkey 23 the parasite had disappeared from the blood, owing probably to the development of a blood immunity, but persisted in the lymphatic glands and spleen, while in Monkey 29 the blood and nodular tissue contained it but the organs were all free

TABLE 1.

M. rhesus No.	Blood		Liver		Spleen		Lymph gland		Bone marrow		Nodule	
	Anemia	Culture	Degree of histological change	Culture	Degree of histological change	Culture	Degree of histological change	Culture	Degree of histological change	Culture		Culture
Group 1 (fatally infected) { 17	+++	+	+++	Cont.	+++	+	+++	+	+++	+	Present	+
25	++++	Cont.	++++	"	+++	Cont.	+++	Cont.	++++	Cont.	"	+
30	++	+	+++	+	+	+	+++	++	+	+	"	+
35	+	++	++	+	+++	++	+	+	+++	++	"	+
37	++++	Cont.	+++	Cont.	+++	Cont.	+++	Cont.	+++	Cont.	"	+
Group 2 (sacrificed during active stage of infection) { 4	⋮	++	−	⋮	+	⋮	++	⋮	⋮	⋮	"	+
5	+	+	−	−	++	+	++	+	++	+	"	+
23	−	−	++	Cont.	++++	+	+++	+	++	+	"	+
24	·++	++	++	+	++	+	+	+	⋮	+	"	+
29	+		−	−	++ −?	−	<+	+		⋮	"	+
Group 3 (sacrificed during convalescence) { 7	−	+	−	Cont.	<+	+	<+	+	⋮	+	Had disappeared	
8	−	−	−	−	−	−	<+	+	−	−	"	
10	−	−	−	−	−	−	−	−	−	−	"	
13	−	−	−	−	−	−	−	−	−	−	"	

Anemia ++++ = r.b.c. 1,000,000 to 1,500,000, Hb. 15 to 25 per cent; +++ = r.b.c. less than 3,000,000; ++ = r.b.c. less than 4,000,000; + = less than 5,000,000; − = over 5,500,000.

Degree of change in tissue varies from marked (++++) to slight (+).

Cont. = secondary bacterial contamination.

Summary of Pathological

M. rhesus No.		Liver
17	Inoculation Mar. 15, 1926. Died after 57 days. Fever still present. Nodules active. Anemia marked (r.b.c. 3,112,000, Hb. 50 per cent). Blood culture + at autopsy. Nodule culture + at autopsy	Pale, somewhat enlarged. Structure disturbed, cells along central zones poorly stained. Perivascular proliferation moderate. Greenish pigment present. *B. bacilliformis* found in a few endothelial cells. Culture contaminated
25	Inoculation Mar. 23, 1926. Died after 36 days. Course practically afebrile. Local lesions extensive, partly generalized. Anemia very severe (r.b.c. 1,624,000, Hb. 25 per cent). Blood (1:100,000) culture + 12 hrs. before death. Culture of nodules taken at autopsy +	Pale, soft, mottled, perhaps enlarged. Structure fairly well preserved but pronounced central necrosis throughout. Numerous phagocytes, polymorphonuclears, and pigments present. Hepatic cells vacuolated. Culture contaminated
30	Inoculation Mar. 31, 1926. Died after 25 days. Fever present. Nodules small but active. Blood (1:10,000 dilution) culture + 3 days before death. Anemia moderate (r.b.c. 3,920,000, Hb. 60 per cent). Blood and nodules taken at autopsy yielded cultures	Pale, appears fatty. Normal structure lost, cells stain poorly, vacuolated and dissociated. Endothelial hyperplasia; some cells contain *B. bacilliformis*. Many phagocytes in sinusoids. Culture +
35	Inoculation Apr. 12, 1926. Died after 29 days. Fever still present. Nodules small but active. Anemia slight (r.b.c. 4,776,000, Hb. 80 per cent). Blood (1:100) culture + 10 days before death. Blood and nodules taken at autopsy yielded cultures	Probably paler than normal. General structure disturbed. Marked dissociation and vacuolation of cells. Invasion of sinusoids by phagocytes. Proliferation of endothelial cells. Culture +

and Cultural Findings.

Spleen	Lymphatic glands	Bone marrow	Remarks
Enlarged, rather firm. Follicles reduced. Endothelial hyperplasia. Active phagocytosis. Fibrin masses and pigments present. *B. bacilliformis* found. Culture positive	All lymph nodes much swollen and congested. Endothelial hyperplasia and active phagocytosis throughout. Culture +	Dark grayish red. Very active phagocytosis. Numerous normoblasts and polymorphonuclear leucocytes present. Culture +	*B. bacilliformis* found in all tissues studied
Slightly enlarged, soft. Follicles smaller, outlines of many lost. Numerous perifollicular necrotic foci. Marked endothelial hyperplasia. Active phagocytosis of degenerated polymorphonuclears and erythrocytes. Many normoblasts present. Culture contaminated	Marked swelling. throughout. Mesenteric nodes appear like scattered or clustered peas but bluish in color. Pronounced proliferation of endothelial cells and active phagocytosis. Culture contaminated	Dark grayish red. Active phagocytosis. Polymorphonuclears and normoblasts present. Culture contaminated	Terminal bacterial infection (pericarditis) intervened after heart puncture 24 hrs. before death
Enlarged. Follicles thinned and diffuse in places. Endothelial elements increased around vessels; small foci with fibrinous exudate and cell débris. Culture +	General swelling. Endothelial hyperplasia, active phagocytosis, greenish pigments. *B. bacilliformis* found in a few endothelial cells. Culture +	Grayish red. Active phagocytosis. Polymorphonuclears and normoblasts present. Culture +	*B. bacilliformis* found in all tissues studied
Dark and soft. Follicles ill defined, pervaded by increased endothelial cells. Active phagocytosis. Culture +	Swelling and congestion throughout. Endothelial hyperplasia. Phagocytosis. Culture +	Grayish red. Active phagocytosis. Culture +	*B. bacilliformis* found in all tissues studied

M. rhesus No.		Liver
37	Inoculation Apr. 17, 1926. Died after 41 days. Fever still present. Small nodule on leg. Blood (1:1,000) + 96 hrs. before death; r.b.c. 1,664,000, Hb. 25 per cent. Autopsy blood contaminated; anemia extreme (r.b.c. 1,176,000, Hb. 15 per cent). Nodules (autopsy) +	Pale and soft. General structure much disturbed. Cells stain very poorly, necrotic along central zones. Small masses of polymorphonuclear leucocytes scattered in sinusoids, where many loaded phagocytes are present. Endothelial proliferation marked. Culture contaminated
4	Inoculation Dec. 8, 1925. Killed after 22 days. Fever present. Nodules active. Blood culture at autopsy +. Nodules at autopsy +	Apparently normal. No culture made
5	Inoculation Dec. 11, 1925. Killed after 68 days. Had become afebrile. Nodules large and active. R.b.c. 4,856,000, Hb. 85 per cent. Blood culture and nodules (autopsy) +	Apparently normal. No culture made
23	Inoculation Mar. 9, 1926. Killed after 53 days. Fever present. Nodules active, extensive. Blood + in 1:100,000, 40 days previously, − 22 days previously and at autopsy. Nodules (autopsy) +	General structure irregular; some ill stained foci. Numerous polymorphonuclears. Moderate endothelial hyperplasia. Culture contaminated
24	Inoculation Mar. 19, 1926. Killed after 24 days. Fever present. Nodules active. Anemia moderate (r.b.c. 3,432,000, Hb. 55 per cent). Blood (1:10) and nodules at autopsy +	Pale, mottled. Moderate degree of central necrosis, uniform structure lost in places. Endothelial proliferation around vessels. *B. bacilliformis* found in a few endothelial cells. Culture +

and Cultural Findings.

Spleen	Lymphatic glands	Bone marrow	Remarks
Dark and firm. Only a few follicles preserved. Necrotic areas (infarctions) showing active phagocytosis found. Increase in endothelial cells. Culture contaminated	Swollen and congested. Endothelial hyperplasia. Active phagocytosis. Polymorphonuclear leucocytes present. Culture contaminated	Grayish red. Active phagocytosis and increase in polymorphonuclear leucocytes. Culture contaminated	Terminal secondary infection by strain of *B. paratyphosus* A 48 hrs. before death
Somewhat swollen and firm. Considerable increase in endothelial elements. No culture made	Enlarged and congested everywhere. Definite endothelial hyperplasia; active phagocytosis; pigments. No culture made	Not examined	Pure infection with *B. bacilliformis*
Enlarged, granular surface. Follicles ill defined in many places. A few necrotic areas. Active phagocytosis. Culture +	Moderately swollen and congested. Phagocytosis active. Hyperplasia of endothelial cells. Culture +	Grayish red. Active phagocytosis. Normoblasts present. Culture +	*B. bacilliformis* found in all tissues studied except liver. Animal moderately resistant
Somewhat enlarged, shows numerous brown patches of infarction. Many perifollicular necrotic areas. Active phagocytosis. Pigments present. Endothelial hyperplasia. Culture +	Greatly swollen and congested. Hyperplasia of endothelial elements. Very active phagocytosis. Culture +	Grayish red. Phagocytosis active. Many polymorphonuclear leucocytes. Culture +	Note absence of *B. bacilliformis* in blood and persistence in other tissues
Enlarged, bluish red. Many follicles diffuse, interspaced by proliferated endothelial cells. Active phagocytosis. Many normoblasts. Culture +	Swollen and congested. Endothelial hyperplasia and phagocytosis. Culture +	Grayish red. Active phagocytosis. Numerous polymorphonuclears and normoblasts present. Culture +	*B. bacilliformis* found in all tissues studied. Animal quite susceptible

M. rhesus No.		Liver
29	Inoculation Mar. 30, 1926. Killed after 28 days. Fever had been present for 2 wks. Nodules very active. Blood (1:1,000) + day before autopsy. Anemia noticeable. R.b.c. 4,704,000, Hb. 70 per cent. Blood and nodules at autopsy +	Mottled. Microscopically appears normal. Culture −
7	Inoculation Dec. 21, 1925. Killed after 58 days. Fever had subsided 10 days previously. Nodule removed 30 days previously +. Blood + 8 days previously. Blood taken at autopsy +. R.b.c. 5,472,000, Hb. 80 per cent	Appears normal. Culture contaminated
8	Inoculation Dec. 30, 1925. Killed after 49 days. Fever had subsided 7 days previously. Nodules absent. Blood + 14 days previously, − at autopsy. R.b.c. 5,496,000, Hb. 80 per cent	Appears normal. Culture −
10	Inoculation Jan. 8, 1926. Killed after 40 days. Fever had subsided 14 days previously, nodules 3 wks. previously. Blood (1:100) + 30 days previously, − at autopsy. R.b.c. 6,584,000, Hb. 65 per cent	Appears normal. Culture −
13	Inoculation Jan. 19, 1926. Killed after 30 days. Fever had subsided 10 days previously. No nodules. Blood + 19 days previously, − 8 days previously, − at autopsy. R.b.c. 6,104,000, Hb. 90 per cent	Appears normal. Perivascular infiltration present. Culture −

from it.   In the latter instance the organism had apparently not yet invaded the organs.

Of the four animals in which fever and local lesions had been present but had subsided, two (Monkeys 7 and 8) proved to be still carrying *Bartonella bacilliformis* in one tissue or another.   Monkey 8 is especially interesting, as the latent infection was demonstrable only in the lymphatic glands.   The other two animals (Monkeys 10 and 13) had

and Cultural Findings.

Spleen	Lymphatic glands	Bone marrow	Remarks
Enlarged, bluish red. Follicles appear to be of normal size and structure. Moderate endothelial hyperplasia. Numerous normoblasts. Culture −	Swollen and congested. Hyperplasia of endothelial elements, some phagocytosis. Culture +	Not examined	Liver and spleen had probably not yet been invaded. Animal rather resistant
Enlarged, follicles prominent. Only slight hyperplasia of endothelial cells microscopically. Follicles apparently normal. Culture +	Moderate swelling. Slight endothelial proliferation. Culture +	Grayish red. No sections. Culture +	*B. bacilliformis* found in most tissues studied: evidence of chronicity of infection
Appears normal. Culture −	Marked swelling and congestion. Hyperplasia of endothelial cells. Phagocytosis active. Culture +	Grayish yellow. Apparently normal. Culture −	*B. bacilliformis* found only in lymph nodes. Animal apparently convalescing
Probably enlarged. Microscopically normal. Culture −	Definitely enlarged, but pale. General hyperplasia. Culture −	Grayish yellow. Normal. Culture −	Had recovered from infection mildness of which was probably due to resistance of animal
Slightly enlarged (?); surface uneven, firm. Definite increase in fibrous tissues. Hyperplasia of endothelial cells. Culture −	Considerable swelling and congestion. Hyperplasia. Culture −	Grayish yellow. Apparently normal. Culture −	Recovered from mild infection. High resistance of animal noted

completely recovered, as indicated by the histological findings and proven by the negative results of cultural experiments.

Brief protocols of all the animals of the present series are recorded above.

SUMMARY.

The pathological changes observed in the organs in *Macacus rhesus* monkeys which have succumbed to severe infection with *Bartonella*

bacilliformis are similar to those found in human organs in persons dying of Oroya fever.

The characteristic changes in the liver are the zonal necrosis of the cells around the hepatic veins, involving active macrophagocytosis of invading polymorphonuclear leucocytes in the necrotic areas, and a marked endothelial hyperplasia in the sinusoids or around the portal veins.   In some instances there is fatty infiltration of hepatic cells.

In the spleen persistent hyperplasia of the endothelial cells of the capillaries leads to the formation of minute foci of infarction, owing to occlusion of the lumina.   The follicles are dispersed or reduced, and there is an active macrophagocytosis of cellular débris, polymorphonuclear leucocytes, and erythrocytes.   In some specimens an increase in normoblasts is noted.   Pigment is sometimes present.

The lymphatic system shows general progressive endothelial hyperplasia, with active invasion of macrophages which contain polymorphonuclear leucocytes, erythrocytes, and greenish or dark pigments.

In the bone marrow there is increased activity of macrophagocytes. Numerous normoblasts are found in some instances.

In one monkey, sacrificed during the course of infection, small, verruga-like nodules were found in the lungs and spleen.

Bartonella bacilliformis has been detected microscopically, though in small numbers, in all tissues showing histological changes.   Parallel cultural determinations of the presence of *Bartonella bacilliformis* in the blood, liver, spleen, lymphatic glands, bone marrow, and local lesions established the relationship between the pathological conditions and the presence of the parasite.   The organism seems to persist longest in the lymphatic glands.   Cultural methods offer a simple and conclusive means for the determination of the presence or absence of the infecting organism.

EXPLANATION OF PLATES.

PLATE 11.

Fig. 1. Experimental Oroya fever.   Section of liver from *M. rhesus* 25, showing a rather characteristic zonal necrosis of the cells around the hepatic veins. Giemsa's stain.   × 50.

Fig. 2. The same section, × 182.

Fig. 3. Human Oroya fever.   Section of liver from the case (S. A. 15) from

which the strain of *Bartonella bacilliformis* employed in these investigations was isolated. The necrotic changes of the liver cells are more diffuse than in the experimental infection, but there is a definite tendency to a central type. Giemsa's stain. × 50.

FIG. 4. The same section, × 182.

FIG. 5. Human Oroya fever.   Section of liver from the collection of the Harvard School of Tropical Medicine.   Courtesy of Professor R. P. Strong.   This patient was free from any verruga lesions and was regarded by the Harvard Commission as a pure case of Oroya fever.   The section shows the characteristic central necrosis.   Note the striking resemblance between this preparation and that of the monkey liver in Fig. 1.   × 50.

FIG. 6. The same section, × 182.

PLATE 12.

FIG. 7. Experimental Oroya fever.   Section of spleen from *M. rhesus* 23.   A splenic follicle in an infarction zone, showing a perifollicular necrosis and the invasion of the follicle by proliferating endothelial cells, endothelial phagocytes, and polymorphonuclear leucocytes.   Giemsa's stain.   × 182.

FIG. 8. Experimental Oroya fever.   Section of spleen from *M. rhesus* 25.   An area of infarction where the splenic nodule has practically disappeared. Giemsa's stain.   × 182.

FIG. 9. Human Oroya fever.   Section of spleen, for comparison.   A splenic follicle within the infarction, showing a general disorganization, necrosis, and phagocytic invasion.   Giemsa's stain.   × 182.

FIG. 10. Human Oroya fever.   Section of spleen from another case.   The structure of spleen is greatly disturbed, shows general endothelial hyperplasia, and the presence of macrophagocytes and polymorphonuclear leucocytes.   Note the striking resemblance between these lesions and those shown in Figs. 7 and 8. Giemsa's stain.   × 182.

FIG. 11. Section of one of the nodules in the lung of *M. rhesus* 23.   Giemsa's stain.   × 50.

FIG. 12. The same section, × 182, showing the proliferation of endothelial cells within the nodule.

PLATE 13.

FIG. 13. *Bartonella bacilliformis*, colonies from a blood agar slant grown for 6 days 25°C.   Smear preparation stained with Giemsa's solution for 1 hour, showing the pleomorphism of the organism.   Inserted here for comparison with the intracellular forms.   × 1,000.

FIG. 14. Film preparation from a nodule in the lung of *M. rhesus* 23, showing irregular masses of *Bartonella bacilliformis* within the cytoplasm of an endothelial cell.   Giemsa's stain.   × 1,000.

FIG. 15. Film preparation from the bone marrow (femur) of *M. rhesus* 23,

showing *Bartonella bacilliformis* within one of the endothelial cells. Giemsa's stain. × 1,000.

FIG. 16. Film preparation from the spleen of *M. rhesus* 23, showing a cell (endothelial?) containing several small masses (*Bartonella bacilliformis?*). Giemsa's stain. × 1,000.

FIG. 17. Section of an experimental skin nodule produced in *M. rhesus* with a strain of *Bartonella bacilliformis* from verruga,[11] showing a dense mass of the parasites within an endothelial cell. Giemsa's stain. × 1,000.

FIG. 18. Section of an experimental subcutaneous lesion produced in the chimpanzee[3] with the strain of *Bartonella bacilliformis* from Oroya fever, showing irregularly scattered masses of *Bartonella bacilliformis*. Giemsa's stain. ×1,000.

FIG. 19. Section of a skin lesion experimentally produced in an ourang-utan,[3] showing a small mass of *Bartonella bacilliformis* (below) and a dense mass of granules, probably of a mast cell (above). Giemsa's stain. × 1,000.

FIG. 20. Human Oroya fever. Section of lymphatic gland, showing two swollen endothelial cells, loaded with minute granules, projecting into the lumen of a capillary vessel. Giemsa's stain. × 1,000. From Case S. A. 15.

FIG. 21. Human Oroya fever. Section of lymphatic gland, showing the swollen endothelial cells lining a capillary vessel in an oblique section. These cells contain the very minute granules first described by the Harvard Commission as an intracellular phase in the life cycle of *Bartonella bacilliformis*. (Courtesy of Professor Strong.) Giemsa's stain. × 1,000.

PLATE 14.

FIG. 22. The lungs of *M. rhesus* 23, showing the pale grayish nodules on both lungs. They measured 2 to 3 mm. in diameter. In the middle, near the trachea, is a grayish lymph node of the size of a pea. Natural size.

FIG. 23. The spleen of the same monkey, showing numerous infarctions. Natural size.

FIG. 24. *Bartonella bacilliformis* in an endothelial cell in a film preparation from one of the nodules in the lung of *M. rhesus* 23. Giemsa's stain. × 1,750.

FIG. 25. *Bartonella bacilliformis* in an endothelial cell. Film preparation from the spleen of *M. rhesus* 23. Giemsa's stain. × 1,750.

FIG. 26. *Bartonella bacilliformis* (?) in an endothelial cell of the bone marrow (femur) of *M. rhesus* 24. Film preparation. Giemsa's stain. × 1,750.

FIG. 27. *Bartonella bacilliformis* in an endothelial cell of the lymph gland of *M. rhesus* 29. Section. Giemsa's stain. × 1,750.

[11] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.
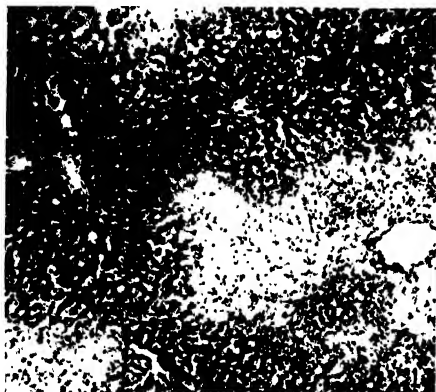
Liver, *M. rhesus* 25. × 50.
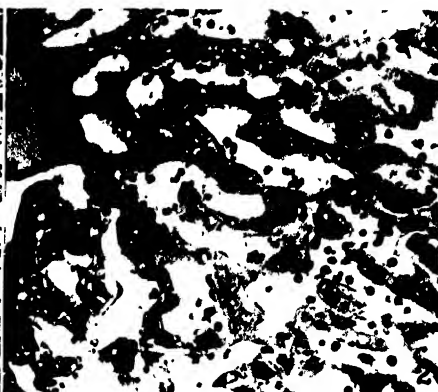
Liver, *M. rhesus* 25. × 182.
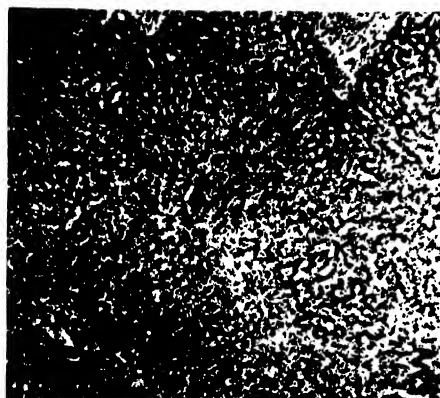
Liver, human Oroya fever. × 50.

Liver, human Oroya fever. × 182.

Liver, human Oroya fever. × 50.

Liver, human Oroya fever. × 182.

(Noguchi: Etiology of Oroya fever. VI.)
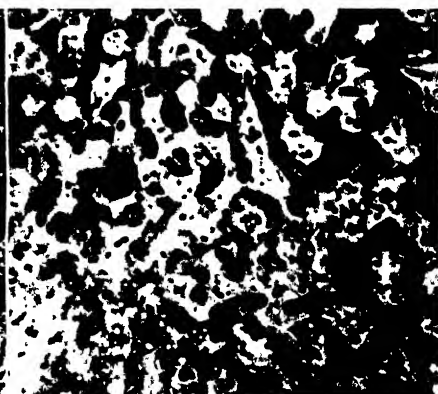
Spleen, *M. rhesus* 23.   × 182.
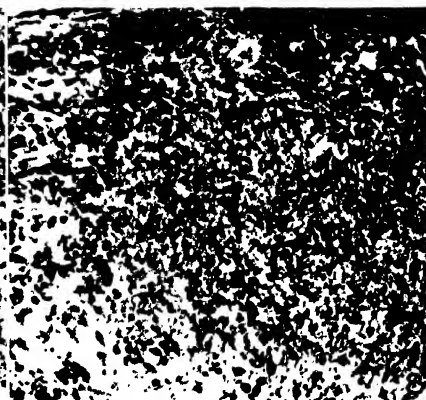
Spleen, *M. rhesus* 25.   × 182.
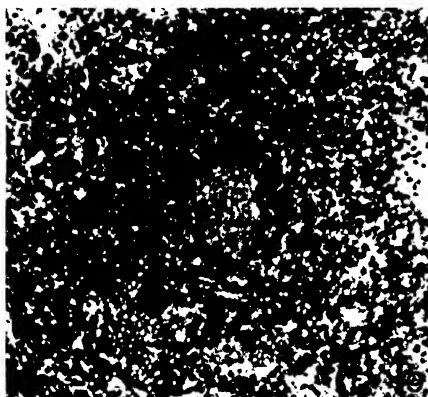
Spleen, human Oroya fever.
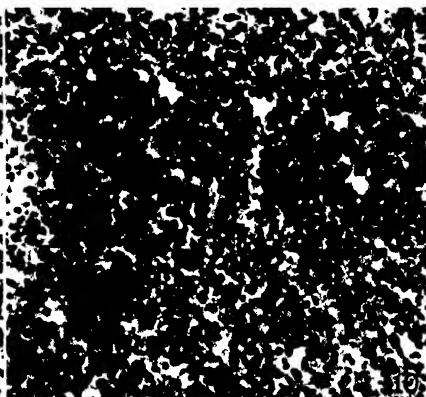
Spleen, human Oroya fever.

Lung nodule, *M. rhesus* 23.   × 50.

Lung nodule, *M. rhesus* 23.   × 182.

(Noguchi: Etiology of Oroya fever.   VI.)
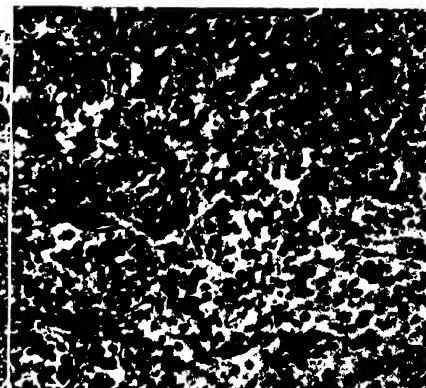
Bartonella bacilliformis. Giemsa's stain.   × 1,000.

In culture, for comparison.

In lung nodule, *M. rhesus* 23.

In bone marrow, *M. rhesus* 23.

In spleen, *M. rhesus* 23.
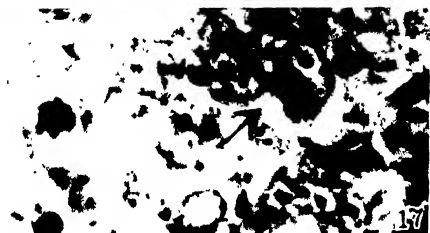
In skin nodule, *M. rhesus.*

In skin nodule, chimpanzee.

In skin lesion, ourang-utan.   In human lymphatic gland.   In human lymphatic gland.

Lesions in lungs and spleen, *M. rhesus* 23.
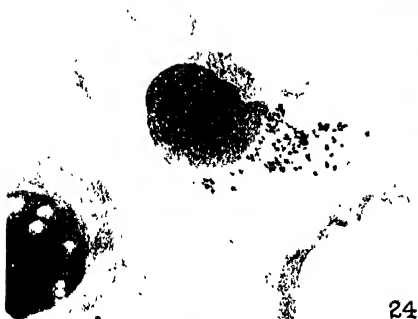
Bartonella, lung nodule, No. 23.
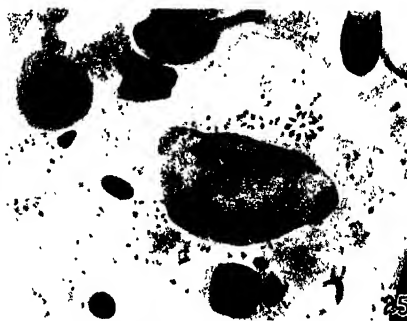
Bartonella, spleen, No. 23.

Bartonella (?), bone marrow, No. 24.
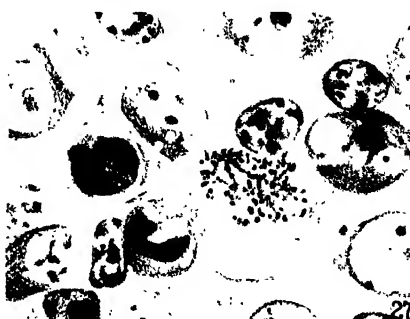
Bartonella, lymph gland, No. 29.

ETIOLOGY OF OROYA FEVER.

VII. The Response of the Skin of Macacus rhesus and Anthropoid Apes to Inoculation with Bartonella bacilliformis.

By HIDEYO NOGUCHI, M.D.

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In animals experimentally infected with *Bartonella bacilliformis*,[1-4] spontaneous involvement of the skin is far less frequent, and rarely so extensive, as in human verruga. In man the organism probably enters the body through the skin, and, after a preliminary multiplication elsewhere—perhaps in the lymphatic glands—invades the skin from within, giving rise to a persistent endothelial hyperplasia. In animals, when *Bartonella bacilliformis* is introduced locally, a lesion arises which may in time assume dimensions as great as the human verruga nodule. But notwithstanding the persistence of the lesion for many months, and the fact that the surrounding skin is frequently bathed in blood resulting from mechanical injury to the lesion, no new nodules develop. Hence it becomes of interest to determine whether the resistance of the skin in monkeys is due to an anergic condition, such as exists in syphilis, or to inability of the parasite to invade the intact skin.

Two other questions presented themselves in this connection, (1) whether it would be possible to induce verruga formation by injuring an area of normal skin in an animal having active skin nodules elsewhere and carrying *Bartonella bacilliformis* in the blood, and (2) whether autoinoculation would be successful in actively infected

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[1] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

[2] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

[3] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 715.

[4] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 729.

animals, *i.e.*, whether skin immunity develops during the course of infection, and if so, at what time.

Resistance of the Intact Skin to Bartonella bacilliformis.

The question whether *Bartonella bacilliformis* is capable of inducing lesions when applied to the surface of the intact skin is one of practical importance.   To test the point, we applied to intact areas of skin whatever material was being inoculated elsewhere.   The infectious material was rubbed on two separate areas, each several cm. square, and usually near the sites of inoculation by scarification or intradermal injection. No cutaneous lesions were ever induced by this method, though lesions invariably developed at the sites of the intradermal injections and in many instances also on the areas inoculated by scarification.

Although unusually extensive lesions (*verruga mular*) are often produced by application of infectious material to scarified areas of the skin, there have been many instances in which this mode of inoculation failed, while typical lesions (*verruga nodular*) always arose at the sites of intradermal injection.   Hence it is desirable always to use both methods of inoculation.

Chimpanzee,[3] *Pan leucoprymnus*, about 4 years old.   *Jan. 19, 1926*, abdominal skin on the right side was shaved and two areas, each about 3 × 4 cm. square, were scarified and smeared with a saline suspension of the skin nodule* of *M. rhesus* 3.   At the same time two adjacent unscarified areas were rubbed with the same suspension.   *Mar. 1, 1926*, pinkish, raised indurated lesions had become noticeable on both scarified areas.   *Bartonella bacilliformis* was demonstrated in the lesions, both in sections and by culture.   No lesions developed on the areas which were not scarified before being smeared with the suspension.

Ourang-utan,[3] *Pongo pygæmus*, about 3 years old.   *Apr. 17, 1926*, abdominal skin shaved and an area (2 × 3 cm.) on the right lower quadrant scarified and rubbed with a piece of nodule from the eyebrow of *M. rhesus* 23.   An unscarified area on the upper quadrant was vigorously rubbed with the same material. *May 20, 1926*, pinkish, raised, linear lesions, rather pale, have been present on the scarified area for some time.   *Bartonella bacilliformis* was demonstrated in the lesions both by culture and in sections.   No lesions developed on the unscarified smeared area.

M. rhesus 18.[2]   *Feb. 15, 1926*, right eyebrow and skin of right abdomen shaved and scarified and a saline suspension of the nodule of *M. rhesus* 5 applied.   Adja-

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* Nodules were removed under ether anesthesia.

cent unscarified areas were rubbed with the same material. Extensive nodules of deep red color developed on the scarified area of the eyebrow within a few weeks and numerous punctiform red miliary nodules on the scarified abdominal skin. The abdominal lesions disappeared within a month, the nodules on the eyebrow remained until the middle of June, when they became small, pale, and fibrous. *Bartonella bacilliformis* was demonstrated in the lesions both by culture and in sections. No lesions developed on the unscarified smeared areas.

M. rhesus 23.[5]   *Mar. 9, 1926*, left eyebrow shaved and a scarified area smeared with a piece of the nodule from the eyebrow of *M. rhesus* 18. An adjacent unscarified area was vigorously rubbed with the same piece of tissue. Within 2 to 3 weeks large protruding lesions had appeared on the scarified area, and these progressed to considerable size during the following weeks. *Bartonella bacilliformis* was found in the sections of the lesion. No lesion appeared outside of the scarified area.

Relation of Injuries to Localization of Lesions.

Whether or not mechanical factors—friction, exposure to minor injury—are involved in the spread of the skin lesions in human verruga is not known. In animals these devices appear to have no influence, as shown by repeated failure to induce localized lesions in infected animals by scarifying the skin or introducing foreign substances (agar, culture medium) intradermally during the height of the infection, when *Bartonella bacilliformis* was demonstrable in the blood. In one unusually susceptible animal, *M. rhesus* 25,[2] in which spontaneous miliary nodules developed, scarification of the abdominal skin had no influence on the course of the general eruption. Similar attempts to induce nodule formation failed also in the chimpanzee, the ourang-utan, and in *M. rhesus* 18,[2] which had at the sites of scarification on the eyebrow one of the most extensive local lesions (*verruga mular*) observed in the course of the experimental work with *Bartonella bacilliformis*.

It is evident that in experimental animals *Bartonella bacilliformis* cannot be made to localize in an area of skin injured either by scarification or by intradermal inoculation of foreign substances, though present in the circulating blood and in the lymph channels, as well as in the skin lesions. Injuries of the skin are not equivalent to the deposition of concentrated infectious material on the scarified skin or in the cutaneous tissues.

[5] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 437.

Autoinoculability and Superinfection.

In a disease like verruga peruana, which persists over a long period, there must be a steady process of self-infection. The manner in which the cutaneous lesions spread has not been definitely determined, but it is certain that the skin must retain its susceptibility during periods of remission and relapse. The skin of *Macacus rhesus* and that of the apes is relatively so insusceptible to infection with *Bartonella bacilliformis* that spontaneous eruptions (*verruga miliar*) seldom occur, but it has been possible to induce well marked nodular lesions (*verruga nodular*) in actively infected animals by intradermal inoculation of suspensions of nodular tissue either from the same or from another animal. Such nodules never attained the size of the primary lesions, and they usually began to recede earlier. In no instance was it possible to induce any lesions on scarified areas during the course of infection, though the materials employed induced typical lesions in control animals.

In infections with the strain of *Bartonella bacilliformis* derived from Oroya fever, the existence of active lesions considerably reduced the susceptibility of the skin to subsequent inoculation. Moreover, once the animals were free from *Bartonella bacilliformis*, they were completely refractory to reinoculation, hence the reduction in susceptibility during the course of illness is due to a partial immunity, not to a state of anergy.

On the other hand, in infections with the strain of *Bartonella bacilliformis* from a case of verruga,[6] there have been exceptional instances in which a preexisting infection did not prevent the development of the most severe type of local infection after reinoculation with the same (verruga) strain. *M. rhesus* 33 and *M. rhesus* 34 showed this type of reaction.

M. *rhesus* 33 was inoculated on Apr. 7, 1926, with the saline suspension of a human verruga nodule (Case P 5). Blood taken on Apr. 16 and again on May 27 yielded (in 1 : 10 dilution) cultures of *Bartonella bacilliformis*.

On June 3 the animal was inoculated intradermally and by scarification with the suspension of a nodule from the eyebrow of *M. rhesus* 41 (second generation of the same strain). On June 24 the lesions had already developed at the sites of inoculation, and by July 1 they were very large and active.

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[6] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

M. rhesus 34 was inoculated on Apr. 10, 1926, with the saline suspension of the nodule of Case P 5. Cultures was obtained from diluted (1:10) blood on April 28, May 12, and May 27. By June 29 a large nodule had developed on the tail.

On June 3 the animal was inoculated intradermally and by scarification with the suspension of the eyebrow nodule of *M. rhesus* 41. *Bartonella bacilliformis* was cultivated'from a 1:100 dilution of blood taken on June 20. Large lesions had appeared at the sites of inoculation at this time, and by July 1 the lesions had become unusually extensive.

As these instances show, it is not possible to make any general statement with regard to the partial immunity observed in most animals when passing through a protracted course of infection with *Bartonella bacilliformis*. Immunity may develop so slowly in some animals as not to interfere with the course of a superinfection.

Pathogenesis of the Skin Lesions.

The verruga-like skin lesion produced by *Bartonella bacilliformis* in anthropoid apes and *M. rhesus* is a slowly progressing infectious angioendothelioma and owes its origin to a delicate reciprocal relation between the endothelial cell and the microorganism. *Bartonella bacilliformis* finds within the cytoplasm of endothelial cells most favorable conditions for its multiplicative phase, and once lodged there multiplies steadily, though perhaps rather slowly, eliciting certain reactions on the part of the infected cells. Since these parasitized cells remain alive for a long time and undergo active mitosis, it is clear that the parasite furnishes a mild type of stimulus which leads to continuous proliferation of the cells. The lesion thus started continues to grow until local as well as general immunity intervenes to terminate the peculiar association of cells and parasite.

The evolution of the verruga lesion in experimental animals may be divided into (1) the incubation period, (2) the initial stage, (3) the mature vascular stage, and (4) the regression. These divisions are of course arbitrary, one stage passing into the next imperceptibly, yet each at its height has its special feature.

Incubation Period.—No instance has been observed in which the lesion was macroscopically recognizable within less than 10 days after inoculation. Usually a trace of induration can be detected after 10 to 14 days, though in some cases the incubation period is as long as 3 weeks.

Initial Stage.—When the nodular lesion, which becomes noticeable as a slightly raised area, 1 to 2 mm. in diameter, has reached a size of 4 to 6 mm. and protrudes 2 to 3 mm. beyond the surrounding normal skin, the color becomes slightly pinkish, and edema of the surrounding tissues becomes evident. The nodule is now firm and well defined to the touch and can be easily separated from the overlying skin and the loose connective tissue in which it is embedded. The cut section is grayish pink, edematous, but not soft, and microscopically the nodule is seen to consist of masses of endothelial cells, placed close together, lying among capillary vessels and connective tissue fibers. A small number of lymphocytes and polymorphonuclear leucocytes are seen in the interstices of the cutis, but not within the nodule itself. The endothelial walls of the capillaries are thickened, and the lumina of the blood vessels narrowed. Practically every endothelial cell in the nodule contains *Bartonella bacilliformis*, some cells being packed with clumps of organisms. The endothelial cells lining the capillaries are often filled with similar masses of microorganisms. The histological picture at this stage is very simple.

Mature and Vascular Stage.—The interval from the preceding stage to this one varies from a few to 10 to 14 days and persists for 10 days to 2 weeks.

The increase in size of the nodule may be gradual or even relatively rapid until the lesion measures 8 to 10 mm. or more in diameter. The color first becomes bright, then deep, red. The overlying skin is shiny, and the epidermis scaly in places. The nodule is still firm and is sharply demarcated from the surrounding tissues. The color may change to a dark bluish red, suggesting that of a cherry. This type of lesion, especially when deep seated and less vascular on the surface, is characteristic of the form of human lesion known as *verruga nodular*. Occasionally the overlying skin yields to the increasing pressure from within, and the lesion becomes pedunculated.

The lesions found on the scarified lines of the skin are seldom round; on the contrary, they are usually irregular at the surface, and the mass resembles a raspberry in form and color, being made up of a number of small, translucent, red, drupelet-like nodules. The entire region adjacent to the lesion is edematous. The lesion is devoid of an epidermic layer, hence it bleeds readily on slight friction. This type of lesion corresponds to that termed in man *verruga mular*.

In a few animals spontaneous nodules have appeared on the skin at sites remote from those inoculated; these correspond to the general eruption in human cases known as *verruga miliar*. Such lesions have always been small, none becoming larger than 1 to 2 mm. in diameter, but their clinical course is similar to that of the nodules induced by intradermal inoculation or by scarification.

On microscopic examination, nodules or lesions removed during the mature and vascular stage present a somewhat more complex picture than those of the initial stage. The large number of capillary vessels intersecting dense masses of closely packed endothelial cells and the increase of fibrous connective tissue irregularly penetrating the nodular structure are striking features of the lesions. Numerous fibroblasts and fibrils lie between islands of angioblasts, the appearance somewhat suggesting that of a fibrosarcoma. In places there is hyperplasia of the epidermis, and some large detached masses of epithelioid cells find their way deep into the nodular zone, a circumstance which complicates the interpretation of the cellular elements composing the nodule. Some of these invading epithelioid cells show hydropic degeneration. Degenerating endothelial cells and numerous erythrocytes are present in some places and are taken up by migrating macrophages, which also engulf the dead polymorphonuclear leucocytes simultaneously present in these areas. Along the interstitial spaces penetrated by connective tissue fibers, plasma cells and mast cells are sometimes rather numerous. The lumina of the capillary vessels are often occluded or compressed by proliferating endothelial cells. Mitotic figures are common. The skin may be adherent.

The microorganisms are more uniformly present in the endothelial cells along the periphery of the nodule than among the cells in the older foci. None except endothelial cells contain *Bartonella bacilliformis* in the cytoplasm, and no extracellular localization of the parasites can be detected.

When a secondary bacterial invasion has taken place, there are found, needless to say, an enormous number of polymorphonuclear leucocytes loaded with the contaminating bacilli or cocci. At times these bacteria-carrying leucocytes are taken up by endothelial cells and give rise to a confusing appearance, as though the secondary invaders were the primary cause of the hyperplasia.

Regression.—The time of beginning of the regressive process depends on the individual animal's resistance to the infection, and the process of regression is intimately associated with the acquired immunity, local or constitutional.   Early regression, before full development of the lesion, has been rather frequent in the experimental animals.   Once the nodule has fully developed, however, considerable time is required for its complete resolution, 4 to 5 months in some instances.   The first indication of retrogression is the gradual loss of the deep red color.   As the color fades the size of the nodule begins to diminish, the paling and diminution continuing until finally only a tiny colorless wart remains. When the nodule ruptures spontaneously at the beginning of the period of regression, the whole mass sloughs and the wound heals rapidly with scar formation.   The result is quite different, however, when the nodule is injured, or partially removed by surgical operation during its developing stages; in this instance the lesion acquires renewed vigor and spreads far beyond its previous limits in the form of a *verruga mular;* it becomes malignant.

The histological appearances of the lesions, as just presented, are similar to those observed by the Harvard Commission[7] in human and experimental verruga tissues, of which they carefully studied a large number.   The eosin-staining inclusions so constantly encountered in the endothelial cells, both in human and monkey nodules, and particularly emphasized by the Commission, are of considerable significance.   These reddish granules, which may have been identical with those described by Mayer, Rocha-Lima, and Werner[8] as Chlamydozoa, in all probability represent intracellular forms of *Bartonella bacilliformis*, somewhat less distinctly stained than in our preparations. The possibility of indistinct staining is one which cannot be ruled out in connection with this difficultly staining microorganism.

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[7] Strong, R. P., Tyzzer, E. E., Sellards, A. W., Brues, C. T., and Gastiaburú, J. C., Report of first expedition to South America, 1913, Harvard School of Tropical Medicine, Cambridge, 1915.

[8] Mayer, M., Rocha-Lima, H., and Werner, H., *Münch. med. Woch.*, 1913, lx, 739.

SUMMARY.

Bartonella bacilliformis failed to induce lesions when merely rubbed on the surface of the intact skin of a chimpanzee, an ourang-utan, and numerous *Macacus rhesus* monkeys, although when applied to the scarified skin of the same animals it gave rise to extensive lesions.

Application of infectious material to the scarified skin did not always induce verruga lesions, but intradermal inoculation almost invariably gave rise to nodule formation.

The localization of *Bartonella bacilliformis* in the skin is not, in experimental animals, determined by mechanical factors, since scarification of the skin or intradermal injection of foreign substances in monkeys infected with *Bartonella bacilliformis* does not give rise to verruga formation.

The degree of susceptibility of the skin tissues appears to be considerably diminished during the course of experimental infection with *Bartonella bacilliformis*. Inoculation of the scarified skin of infected animals gave uniformly negative results, and intradermal inoculation induced only a mild local reaction. In a few exceptional instances, however, of animals previously infected with the strain of *Bartonella bacilliformis* derived from a human verruga nodule, reinoculation with the same strain gave rise to unusually marked reactions.

The evolution of the skin lesion induced in experimental animals by *Bartonella bacilliformis* may be divided into four stages, the period of incubation, the initial stage, the mature and vascular stage, and the regression. In the initial stage the lesion is a pure angioendothelioma, but in the stage of full development the histological picture is complicated by connective tissue proliferation and occasionally also by penetration of epidermis into the lesion. The demonstration of *Bartonella bacilliformis* in the endothelial cells distinguishes the lesion from others which simulate it.

The cutaneous lesions known as *verruga nodular*, *verruga mular*, and *verruga miliar* have been reproduced in monkeys.

EXPERIMENTS ON THE PRODUCTION OF WASSERMANN REAGINS BY MEANS OF TRYPANOSOMES.

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According to the original view of the discoverers the Wassermann reaction was considered, in analogy to the common serological reactions, as being caused by antibodies specific for the infecting microorganisms.   This view became doubtful and was abandoned by most authors when it was shown that syphilitic sera react with alcoholic extracts of organs, presumably with lipoids present in these extracts (1–3).   The use of extracts of normal instead of syphilitic organs (1) has since been accepted almost generally for the technique of the Wassermann reaction.   Several main hypotheses have been advanced in order to explain the formation and properties of the Wassermann reagins.   Some authors believed that the action of the syphilitic sera was due not to immune bodies but to non-specific changes of the serum proteins.   Others regarded the reagins as antibodies formed either by autoimmunization with lipoidal substances from the infected organism or with lipoids derived from the spirochetes (*cf.* Bergel).   In the latter case one had to assume that the antibodies produced act not only upon the homologous antigen but also upon lipoids of very different origin.

The hypotheses implying an immunization against lipoids met with the difficulty that the attempts to induce the formation of antibodies by injection of lipoids, as a rule, did not yield clear-cut and consistent results.   In the positive experiments recorded there was no certainty as to the absence of small quantities of antigenic proteins in the material injected or else the amount of antibody formation was rather insignificant.[1]

[1] Regarding the antigens of blood corpuscles see "On the antigens of red blood corpuscles.   The question of lipoid antigens," by K. Landsteiner and J. van der Scheer, *J. Exp. Med.*, 1925, xli, 427.

A new impulse was given to the study of lipoid antigens by the work on the Forssman's heterogenetic reaction. It led to the view that Forssman's antigen has a complex constitution and is composed of a specific part binding but not immunizing, which part can be extracted by alcohol, and of a protein responsible for the immunizing effect (4–6). Moreover by means of a special method regular results in the immunization with alcoholic extracts of Forssman's antigen (horse kidney) could be obtained (5, 7). These extracts became antigenic when mixed with protein solutions such as human or pig serum, although in themselves they lack this property or exhibit it to a very small degree only. The case of the Forssman antigen is not exceptional as was shown by the application of the same method to the alcoholic extracts of erythrocytes (8). Evidently the findings reported suggested the view that the antigen operative in the production of Wassermann reagins may possibly be of a complex structure analogous to that of the heterogenetic antigen (5, p. 305).

The statement made by Taniguchi (9) that animals treated with Forssman's antigen may develop Wassermann reagins beside the hemolytic antibodies offered no satisfactory explanation since organs free from Forssman antigen did not produce a like effect in his experiments.

Sachs, A. Klopstock and Weil (10) emphasized a hypothesis related to that of Citron. They assume that the change in the serum is due to the action of a combination of lipoids of the tissues with proteins of the spirochetes. The authors actually were able to show that, in analogy to the experiments on heterogenetic antigen, after injections of alcoholic extracts of rabbit organs along with pig serum into rabbits the sera of the animals became Wassermann-positive.

The idea that the microbes contain the antigen responsible for the characteristic alteration of the serum was examined by F. Klopstock (11) and by ourselves (12). In Klopstock's experiments the Wassermann-positive sera were got by injections with killed *Spirochæta pallida*, in ours with killed *Trypanosoma equiperdum*. The use of trypanosomes for the study of the Wassermann reactions is warranted by the fact that infections with trypanosomes often result in the appearance of a positive Wassermann reaction (13, 14) which is

apparently equivalent to that of syphilitic sera. The occurrence of these reactions in rabbits seems to vary considerably with different strains of trypanosomes according to our experience. (See Browning and McKenzie (15).)

The results of our investigation briefly described in a preliminary communication (12) are given more fully in the present paper.

EXPERIMENTAL.

Rabbits were injected with a suspension of dead trypanosomes (*Trypanosoma equiperdum*) prepared as follows: Citrated blood taken from rats at the height of the infection was diluted with an equal volume of saline and the erythrocytes sedimented by gentle spinning. The supernatant fluid and the resuspended white layer on top of the blood corpuscles were joined and the trypanosomes sedimented by vigorous centrifugalization. The sediment was washed once with about 25 times its volume of saline. A suspension was made with about 4 cc. of saline for every rat used. This turbid fluid was rich in live trypanosomes and contained few blood cells when examined microscopically. After addition of one-twentieth of its volume of 5 per cent phenol it was kept in the ice box overnight. By that time the microbes were largely disintegrated. No infections with trypanosomes occurred upon administration of even large doses of this material.

The strain of trypanosomes employed was of low virulence for rabbits, that is, a very chronic disease developed. The lesions, however, were intense and typical.

In all the experiments special care had to be taken in selecting the animals owing to the frequent occurrence of positive reacting sera in stock rabbits. Therefore the sera were tested with the flocculation method and the complement fixation with cholesterolized beef heart extract and with emulsions of commercial egg lecithin (Merck). The animals were used only if these tests were completely negative, or, in some cases, gave very weak reactions with the Sachs-Georgi method with serum diluted 1:2. In order to pick out enough rabbits for the experiments the selection had to be made from a rather great number of animals.

Wassermann Tests.—To 0.25 cc. of progressively doubled dilutions of inactivated serum starting with 1:5 was added 0.25 cc. of an emulsion of cholesterolized beef heart extract and 0.25 cc. of 1:10 guinea pig serum. After incubation for 1 hour at 37°C. 0.25 cc. of diluted sheep blood immune serum containing 2½ to 3 hemolytic units and 1 drop of 50 per cent sheep blood were added.

The antigen was prepared by extracting beef heart with 5 volumes of alcohol 95 per cent and adding 0.06 of the volume of a 1 per cent alcoholic solution of cholesterol. From this an emulsion was made by adding first 1 volume and after about 10 seconds 4 volumes of saline solution.

Sachs-Georgi Tests.—0.2 cc. of the inactivated serum diluted 1:2 and 1:10 were mixed with 0.2 cc. of the emulsion of cholesterolized beef heart extract. The tubes were kept at 37°C. for 2 hours and at room temperature overnight.

TABLE I.

Injections with Dead Trypanosomes. The Animals Were Tested on the Same Day, after They Had Been Injected 4 to 7 Times.

| No. of animals | Before injection Sachs-Georgi flocculation with serum diluted 1:2 | After injections with dead trypanosomes | | | | | | No. of injections |
| | | Wassermann reaction with cholesterolized beef heart extract | Sachs-Georgi flocculation with serum diluted 1: | | Complement fixation with lecithin | Flocculation of lecithin with serum diluted 1:2 | Complement fixation with alcoholic extract of trypanosomes | |
			2	10				
1	0	0,0,tr,vstr	+++	++	ac,c,c	0	0,0,str,c	5
2	0	0,0,0,d,ac,c	+++	++	c,c,c	0	0,0,0,str,ac	5
3	0	0,0,0,0,str,c	+++++	+++	str,vstr,ac,c	0	0,0,0,0,str	4
4	tr	w,d,str,c*	+*	0	vstr,d,str,ac,c	ftr	0,0,w,ac,c	7
5	0	0,0,0,d,c	+++	+++	ac,ac,c	0	0,0,tr,c	6
6	0	0,0,0,0,str	+++	+++	w,0,w,ac,ac,c	+	0,0,0,0,vstr	4
7	tr	0,0,vstr,c	+++	++±	d,str,c	±	0,0,str,c	5
8	0	d,w,str,ac,c	++	+	c,c,c	0	0,0,ftr,tr,ac,c	7

* Tests after 7 injections. The reactions with this serum were strongly positive after 4 injections.

Lecithin Tests.—For the complement fixation 1 part of a ½ per cent alcoholic solution of egg lecithin Merck was emulsified by fairly rapid addition of 24 parts of saline solution. The fluid is opalescent. The complement fixation tests were made as described above. This way of preparing the emulsion was found necessary to avoid reactions with normal sera.

For the flocculation tests (with inactivated serum 1:2) the emulsion was prepared by rapid addition of 5 parts of saline solution to 1 part of a ½ per cent

TABLE II.

Sera of Rabbits Infected with Trypanosomes Tested 1 Month after the Infection

No. of animals	Before infection	1 mo. after the infection with trypanosomes					
	Sachs-Georgi flocculation with serum diluted 1:2	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:		Complement fixation with lecithin	Flocculation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes
			2	10			
9	0	0,0,0,0,str,c	+++	+±	0,0,0,str,c	++±	tr,d,str,c
10	0	ac,ac,ac,c	+±	0	c,c,c	±	vstr,vstr,ac,ac
11	0	str,str,ac,c	+	ftr	0,0,c	+±	vstr,vstr,ac,ac
12	0	0,0,0,ac,c	+++	+	0,0,ftr,ac,c	++±	tr,tr,str,vstr,c
13	0	str,str,vstr,c	+±	±	w,0,w,ac,ac,c	++	ac,ac,c
14	±	0,0,0,tr,c	++±	+	0,0,0,0,ac,c	++	str,str,vstr,ac,c
15	0	0,0,vstr,c	+	tr	0,0,vstr,c	+±	vstr,ac,ac,c

Normal Rabbits.

A		c,c,c,c	0		c,c,c,c	0	c,c,c,c
B		c,c,c,c	0		ac,c,c,c	0	c,c,c,c

The tests recorded in Tables I and II were made on the same day and with the same emulsions. For comparison the reactions of sera of normal rabbits are recorded.

solution of the lecithin. The readings were taken after 20 hours at room temperature.

Tests with Trypanosome Extract.—1 volume of the packed microbes was extracted 24 hours with 25 parts of 95 per cent alcohol. The filtered solution was emulsified by fairly rapid addition of 5 parts of saline solution and the emulsion was used for complement fixation as in the Wassermann tests.

The strength of the reactions in these tests is indicated as follows: Hemolysin tests—0 = no hemolysis, ftr = faint trace, tr = trace, w = weak, d = distinct,

TABLE III.—*8 Rabbits Were Injected with Dead Trypanosomes on April 24, 30, and May 5, May 5*

No. of animals	Before injection					After 2 injections with dead try			
	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:2	Complement fixation with lecithin	Flocculation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:		Complement fixation with lecithin
							2	10	
16	c,c,c,c	0	c,c,c,c	0	ac,c,c,c	vstr,ac,c	tr	0	ac,ac,c
17	c,c,c,c	0	c,c,c,c	0	c,c,c,c	0,0,w,c	++±	±	c,c,c
18	c,c,c,c	0	c,c,c,c	0	c,c,c,c	d,str,c,c	+	0	ac,c,c
19	c,c,c,c	tr	c,c,c,c	0	c,c,c,c	vstr,ac,c	±	0	c,c,c
20	c,c,c,c	0	c,c,c,c	0	c,c,c,c	0,0,d,c	+	tr	vstr,ac,c
21	c,c,c,c	ftr	c,c,c,c	0	ac,c,c,c	0,0,0,d,c	+++	++	ac,ac,c
22	c,c,c,c	0	c,c,c,c	0	c,c,c,c	0,0,w,ac,c	+++	+	ac,c,c
23	c,c,c,c	0	c,c,c,c	0	c,c,c,c	d,w,vstr,ac	ftr	0	c,c,c,c

Normal Rab

C						c,c,c	0	0	ac,c,c
D									

* Tests 13 days after the 1st injection.

with Doses Corresponding to 6 or 7 Mg. Dry Weight of Trypanosomes.   The Tests Were Made on and 13.

panosomes (after 11 days)		After 3 injections with dead trypanosomes (after 19 days)						
Flocculation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:		Complement fixation with lecithin	Flocculation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes
			2	10			
0	0,w,ac,c	0,0,0,0,0,ftr,ac,c	++	++±	c,c,c,c	0	0,0,0,0,w,vstr,ac
tr	0,0,w,vstr	0,0,0,0,0,0,vstr,ac	+++	+++	c,c,ac,c	0	0,0,0,0,0,w,ac
0	0,tr,vstr,c	0,0,0,0,str,c,c	+++	++±	c,c,c,c	+±	0,0,tr,vstr,c
0	str,vstr,d	0,0,0,0,vstr,c	+++	+±	c,c,c,c	±	0,0,str,ac,c
0	0,0,d,ac,c	0,0,0,0,vstr,c	+±	±	c,c,c,c	0	0,0,d,ac,c
0	0,0,0,str,c	0,0,0,ftr,ac,c	+++	++±	c,c,c,c	0	0,0,0,0,w,c
0	0,0,tr,c	0,0,0,0,0,0,d,ac	+++	+++	c,c,c,c	tr	0,0,0,0,ftr,vstr,c
0	tr,str,c*	str,d,vstr,ac,c	ftr	0	c,c,c,c	0	0,0,0,vstr

bit Sera.

0	c,c,c	c,c,c	0	0	c,c,c	0	c,c,c
		ac,c,c	0	0	c,c,c	0	c,c,c

TABLE IV.

Tests with Sera of 7 Rabbits Infected with Trypanosomes.

The table reproduces the results at the time when the strongest reactions were observed. In the last column the number of days after the infection is given.

No. of animals	Before infection					After infection						Days after infection
	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation with serum diluted 1:2	Complement fixation with lecithin	Flocculation with lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi flocculation 1:2	Sachs-Georgi flocculation 1:10	Complement fixation with lecithin	Flocculation with lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes	
24	c,c,c,c	tr	c,c,c,c	0	ac,c,c,c	0,0,0,0,str,c	+++	+++	0,0,0,ftr,str,ac,c	+++	tr,ftr,0,w,str	32
25	ac,ac,c,c	tr	c,c,c,c	0	c,c,c,c	0,0,0,ftr,vstr	+±	+	0,0,0,tr,ac,c	+	w,w,d,str,ac	32
26	c,c,c	0	c,c,c	0	c,c,c	vstr,ac,c,c	tr	ftr	ac,c,c,c	0	ac,c,c,c	22
27	ac,c,c	0	c,c,c	0	c,c,c	0,0,0,0,vstr,c	+++	+++±	0,0,0,0,c	+++	0,tr,w,vstr,ac,c	50
28	ac,c,c	0	c,c,c	0	c,c,c	d,tr,str,ac	tr	0	w,str,ac,c	+	d,str,vstr,c	22
29	vstr,c,c	0	c,c,c	0	c,c,c	0,0,0,vstr,ac,c	+++	++	0,0,0,str,c	+++±	vstr,str,ac,c,c	42
30	c,c,c	0	c,c,c	0	c,c,c	0,0,0,vstr,ac,c	+±	±	0,0,w,ac,c	++	d,w,vstr,ac,c	42

36

str = strong, vstr = very strong, ac = almost complete, c = complete hemolysis. Flocculation tests—0 = no flocculation, ftr = faint trace, tr = trace; ±, +, ++, +++, etc.

In all experiments positive sera of known strength and negative ones were used as a control.

In our first experiments 7 rabbits were infected by injection of 1 cc., 50 times diluted blood from an infected rat, and 8 rabbits re-

TABLE V.

Human Syphilis Sera.

Nos. of the sera	Wassermann reaction with cholesterolized beef heart extract	Sachs-Georgi tests with serum diluted 1:		Complement fixation with lecithin	Floccu-lation of lecithin with serum diluted 1:2	Complement fixation with alcoholic extract of trypanosomes
		2	10			
S_1	0,0,0,0,w,vstr,c	++++	+++	ac,c,c,c	±	str,d,str,vstr,ac
S_2	0,0,0,0,tr,d,str	++++	+++	c,c,c,c	+	ac,vstr,ac,c
S_3	0,w,vstr,c	±	0	vstr,vstr,ac,c	±	
S_4	0,tr,ac,c	+++	++	c,c,c,c	+	c,c,c,c
S_5	0,0,0,d,c	++	+	c,c,c,c	+±	vstr,ac,c
S_6	0,0,0,d,c	+++	++	c,c,c,c	+	ac,ac,c

Rabbit Syphilis Sera.

50	0,0,0,w,ac,ac	+++	++±	0,0,d,ac,c	++±	tr,str,c,c
51	0,0,w,ac	+++	±	tr,str,c	±	
52	0,0,w,c	++±	++±	0,tr,str,ac	++	
53	0,ac,c,c	++±	ftr	d,str,c	0	
54	0,0,0,tr,vstr,ac	++++		0,tr,str,ac,c	++±	0,0,tr,vstr,ac
55	0,0,tr,vstr,vstr	+++	+	0,tr,vstr,ac,c	+	ftr,ftr,d,vstr,c

ceived every 4 to 7 days intravenous injections of $3\frac{1}{2}$ cc. of an emulsion of killed trypanosomes, containing 6 to 7 mg. of dry material.

Table I shows that with dead trypanosomes the sera of 7 of the 8 animals turned positive in the Wassermann and Sachs-Georgi reaction. In most cases the reactions were strong already after 3 to 4 injections. In one case the serum was weak in the Wassermann reaction although distinctly positive with trypanosome extract and in the Sachs-Georgi reaction. In the infected animals the

results were less regular, especially as regards the Wassermann tests, while the lecithin reactions were positive in all cases but one (Table II).

The changes in the reaction during the course of the experiments and also the differences between the various sort of antigens will be fererred to later.

TABLE VI.—*Rabbits Injected*

No. of animals		11	19	27
	No. of days after the first injection.................	11	19	27
	No. of injections............	2	3	4
16	Wassermann reaction..	vstr,ac,c	0,0,0,0,0,ftr,ac	0,0,0,0,ftr,str,vstr
	Sachs-Georgi with serum diluted 1:2....	tr	++	++±
	Complement fixation with trypanosome alcoholic extract.....	0,w,ac,c	0,0,0,0,w,vstr,ac	0,0,0,0,0,ftr,vstr
	No. of days after the first injection.................	11	19	27
	No. of injections............	2	3	4
17	Wassermann reaction..	0,0,w,c	0,0,0,0,0,0,vstr,ac	0,0,0,tr,str,vstr
	Sachs-Georgi with serum diluted 1:2....	++±	+++	+++
	Complement fixation with trypanosome alcoholic extract.....	0,0,w,vstr	0,0,0,0,0,w,ac	0,0,0,0,tr,str
	No. of days after the first injection.................	11	19	27
	No. of injections............	2	3	4
18	Wassermann reaction..	d,str,c,c	0,0,0,0,str,c	0,0,0,tr,vstr,c
	Sachs-Georgi with serum diluted 1:2...	+	+++	++++
	Complement fixation with trypanosome alcoholic extract.....	0,tr,vstr,c	0,0,tr,vstr,c	0,0,0,0,d,ac

A second experiment was performed similar to the first.

The results presented in Tables III and IV agree well with the foregoing. In the experiment with dead trypanosomes 7 out of 8 animals showed strongly positive Wassermann reactions after 3 injections corresponding to 6 to 7 mg. dry weight 19 days after the first

injection. All the sera reacted positively with trypanosome extract. Frequently the reactions started suddenly. With lecithin no complement fixation was observed while in the former experiment a few sera gave weak reactions. Weak or moderate flocculation reactions occurred in some instances with lecithin. The Sachs-Georgi tests

with Dead Trypanosomes.

34	40	47	53	62
5	6	7	8	9
0,tr,str,ac,c	w,w,vstr,c	ac,c,c	ac,c,c	d,vstr,ac,c
+++	tr	ftr		ftr
		0,d,str,c	0,d,ac,c	tr,str,vstr,c
34	40	47	53	62
5	6	7	8	9
0,0,w,vstr,c	0,0,w,vstr,c	0,tr,str,ac,c	0,tr,str,ac,c	0,d,vstr,c
++++	++±	++±		++
		0,d,ac,c	0,d,ac,c	0,str,vstr,c

ran parallel to the Wassermann reaction. After 2 injections, 11 days from the beginning of the treatment the fixation tests were already positive in 4 out of 8 animals (see Table III) with Wassermann antigen and 6 were positive with alcoholic trypanosome extract. Presumably the reactions with trypanosome extract precede those

TABLE VII.—*Complement Fixation Tests with*

No. of animals		33	47	57
9	No. of days after infection.........	33	47	57
	Wassermann reaction......	0,0,0,0,vstr,c	0,0,0,0,ac	0,0,0,0,0,str,ac,c
	Complement fixation with extract of trypanosomes..	tr,d,str,c	d,tr,w,d	0,0,0,w,vstr,ac c
12	No of days after infection..........	33	47	57
	Wassermann reaction......	0,0,0,0,ac,c	0,0,str,ac,c	w,str,vstr,c
14	No. of days after infection.........	33	47	57
	Wassermann reaction......	0,0,0,tr,c	0,0,0,0,0	0,0,0,0,0,0,d,c
	Complement fixation with extract of trypanosomes..	str,str,vstr,ac,c	d,ftr,0,tr,str	ftr,0,0,0,vstr,ac
24	No. of days after infection.........	17	24	32
	Wassermann reaction......	vstr,c,c	0,0,0,0,tr,vstr,ac	0,0,0,0,0,str,c
25	No. of days after infection.........	17	24	32
	Wassermann reaction......	vstr,c,c	0,0,0,0,ac	0,0,0,ftr,vstr
27	No. of days after infection.........	9	16	22
	Wassermann reaction......	w,d,vstr,ac,c	str,vstr,vstr,ac,c	ftr,ftr,d,ac

Sera of Infected Rabbits at Various Stages.

70	84	98	175	
0,0,0,0,0,ac	0,0,ftr,vstr,ac,c	0,0,0,tr,c	w,d,str	
0,tr,vstr,ac,c	tr,w,vstr,c,c	d,w,ac,c	str,c,c	
70	84	98	175	
ftr,tr,str,c	d,w,vstr,c	0,0,w,ac,c	0,0,0	
70				
0,0,0,0,0,0,vstr				
0,0,tr,vstr,c				
37	43	59	70	145
0,0,0,0,tr,vstr,c	0,0,0,0,0,str,ac	0,0,0,vstr,c,c	tr,d,vstr,c	c,c,c
37	43	56	70	145
tr,0,tr,vstr,c	d,ftr,str,ac,c	ac,c,c	c,c,c,c	vstr,ac,ac
36	50	125		
0,0,0,tr,ac,c	0,0,0,0,0,vstr,c	c,c,c		

with the heart extract in analogy to the findings of F. Klopstock. Regarding these reactions some doubt may arise as to whether or not they are due to a fixation of complement with substances derived from rat cells, since the presence of small quantities of these in the injected material could not be avoided. Control experiments with rat blood extracts however seem to rule out the possibility that such an effect influences the results essentially.

Of the infected animals one failed to develop reagins while in another the response was feeble. Again the reactions with lecithin are well pronounced, those with trypanosome extract are weak. In some cases the latter became strong at a later stage (Table VII, Animals 9 and 14). Positive reactions with alcoholic trypanosome extract are reported by other authors in trypanosomiasis. In this and in the first set of infections the sera gave a strong agglutination when mixed with a suspension of live trypanosomes. The phenomenon was not closely examined. (Rieckenberg's reaction?)

For comparison some tests with sera of human and rabbit syphilis are given in Table V.

It may be of interest to give examples of the changes in the reactions occurring during the course of the experiments (Tables VI and VII).

One sees from the tables that the sera of the animals which were continually injected and also in the infected ones, the antibodies tend to decrease or disappear after a certain time. In some of the infected rabbits kept under observation for several months this drop was followed by a recurrence of the reactions (for instance Rabbit 12).

In order to ascertain that the effects described are peculiar to the antigens contained in trypanosomes, control experiments were performed with other materials as, cholera vibrios, emulsions of beef and pig kidney, and with pig serum, the same amounts (in terms of dry weight) being used for the injections as in the experiments with dead trypanosomes. In each case 4 rabbits were taken. Only in 1 animal did a distinctly positive Wassermann reaction develop after 4 injections with beef kidney.

DISCUSSION.

The main result of the experiments described is the fact that the sera of rabbits become strongly Wassermann-positive upon injections of dead trypanosomes. Flocculation reactions according to Sachs-Georgi corresponded to the complement fixation.

The immunization was obtained with small quantities of antigen and almost regularly. In this respect the findings resemble those of F. Klopstock with *Spirochæta pallida*. Although injections with other materials may occasionally give rise to positive reactions,[2] a peculiar quality of the microorganisms studied must be assumed in view of the ease with which they induce the production of the reagins. Equal amounts of other substances as vibrios, organs and serum used in control experiments yielded almost uniformly negative results. Also the difference in the effects of injections of normal and syphilitic organs as demonstrated by Citron and Munk (18) and recently confirmed by Heimann (19) can be explained more readily by the action of the microorganisms as such than by the interpretation favored by Heimann.

As mentioned before Sachs, A. Klopstock and Weil attribute the appearance of the Wassermann reagins to an autoimmunization against tissue lipoids bound to proteins of the spirochetes. A certain difficulty arises for the idea of Sachs and his coworkers from the fact that the method employed in their experiments calls for an intensive treatment with large amounts of antigenic material (serum). Furthermore their findings do not explain the occurrence of the Wassermann reaction just in cases of syphilis in contradistinction to other infectious diseases unless one assume, as the authors do, an exceptional aptitude on the part of the spirochetes to bring forth the antigenic properties of lipoids. Such an assumption however has not yet been substantiated by experimental data. At first sight the demonstration of a particular antigenic activity of the spirochetes and trypanosomes themselves seems to give a more natural answer to the question of the formation of Wassermann reagins.

[2] See Brandt, Guth and Müller (16), van der Scheer (17), Klopstock, F., *Deutsch. med. Woch.*, 1925, li, 1701. In the latter experiments organs were used of rabbits which had undergone a treatment with dead bacilli.

To be sure, there still remain several aspects of the problem requiring further explanation.[3] If the action of the Wassermann-positive sera on organ lipoids is supposed to be a heterogenetic reaction brought about by antibodies for lipoids of the microbes one should expect the reaction with the latter to be more intense or at least nearly equal in strength to the former. This is true for the experiments of F. Klopstock, and for ours as far as they were performed with injections of killed trypanosomes. On the other hand the reactions which we obtained with the sera of the infected animals were generally much weaker with trypanosome extracts than with the heart lipoids. While the higher dilution of the trypanosome extract, or the fact that it was not cholesterolized, may possibly account for the weaker·reactions it does not explain the difference between the two sorts of experiments, namely infection and immunization with killed microorganisms. Another discrepancy appears in the tests with lecithin (complement fixation and flocculation). These are quite generally much stronger in the case of the infected animals. We found such lecithin reactions also with syphilitic sera of rabbits. Positive lecithin reactions have been recorded by Sachs as a result of the combination method.

Thus one may conclude that the effects are not altogether conformable in both cases; *viz.*, treatment with dead ·trypanosomes and infection.

In our opinion, these differences do not disprove the hypothesis that the microbes are the active agent in the formation of the reagins. The substances derived from the trypanosomes during the infection and those formed in the disintegration *in vitro* may be somewhat different and it is not improbable that such variations are reflected in the response of the animal. Changes in the method of preparing the antigen may suffice to alter the results, and further investigation along this line is desirable.

Still it cannot be stated as yet that the serological changes occurring during the syphilitic infection are due to one single principle. There is for instance not yet a definite explanation for the occur-

[3] As for instance the observations on flocculation reactions with substances other than lipoids. (*Cf.* Weisbach, W., Wassermannsche Reaktion, etc., Jena, 1924.)

rence of positive Wassermann and flocculation reactions in normal animals.

Mention may also be made of the autohemagglutinins found in infections with protozoa and of autohemolysins (paroxysmal hemoglobinuria), apparently specific (20, 21) for human blood in cases of syphilis, though this phenomenon is rarely met with. Perhaps these observations may be indicative of an autoimmunization.

SUMMARY.

Since it is known that positive Wassermann reactions prevail in trypanosomiasis of rabbits, similar to those in syphilis, trypanosomes were used for an inquiry into the cause of this reaction.

Injections with dead trypanosomes into rabbits proved that these microbes are highly active antigens and suffice in themselves to produce strongly positive Wassermann sera, in analogy to the findings reported by F. Klopstock with *Spirochæta pallida*.

Although a number of questions require further study, yet it seems likely that this antigenic activity of the microbes or their products plays a part in the production of the Wassermann reagins in infections with spirochetes and trypanosomes.

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INFLUENCE OF LIGHT ON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

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One of the striking features of the disease induced by a certain transplantable neoplasm of the rabbit is the variability of its course and of the general character of its manifestations (1). Not only do individual animals differ in respect to the plane or level of malignancy but well defined group variations are also observed between series of rabbits inoculated at one time and those inoculated at another. On the whole, this variability is seasonal in character in that the disease is most severe in the spring and fall and is usually milder in the summer than in the winter months (2). The influence of the seasonal factor has been interpreted as operating upon or affecting general animal economy and susceptibility or resistance to disease is considered as a functional activity of the animal organism.

Although many external and internal factors undoubtedly participate in the seasonal variability of this malignant disease, there appears to be a correlation between sunshine and the manifestations and outcome of the condition. That is, the actual hours of sunshine, together with the rate, extent and persistence of change in the curve of sunshine over a given period, can be related to the observed general level of malignancy. The experiments upon which this relationship has been based were carried out with rabbits kept in rooms which receive practically all light diffused through ordinary window glass so that the effects observed cannot be wholly attributed to the shorter ultra-violet rays.

The idea of a correlation between the external factor of light on the one hand, and the physical state and functional activities of the animal organism on the other, has been tested by a series of experiments in which conditions of light could be controlled. The particular

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points studied in beginning the work were the effects produced by a continuous illumination of maximum intensity practicable with simple equipment and by an absence of light, first upon the physical state of normal rabbits as shown by the weights of organs (3) and second, upon the course and character of the malignant disease. The results of the first tumor experiment which were summarized in a preliminary note (4) published soon after the conclusion of the work are here reported in full.

Methods and Material.

The experiment was begun on January 16 and concluded on April 15, 1925. Three animal rooms, with similar environmental conditions other than those of lighting, were employed. These rooms will be referred to as the light, the dark and the control or unaltered room, respectively.

A *constant* source of light was furnished by twelve 1000 watt Mazda lamps and three Cooper Hewitt 50 inch low pressure mercury arcs, Type P, in crown glass, arranged on a rectangular frame in the center of a room from which all other light was excluded. The lamps and arcs were distributed in three horizontal rows 2½ feet apart and each row contained four lamps and one arc. The animal cages were placed parallel with and on either side of the light source at a distance of 3½ feet.

The intensity of light reaching the cages was not entirely.uniform, but on the average was 425 foot candles. The spectrogram of the mercury arc furnished by the Cooper Hewitt Company, shows that the crown glass absorbs all light below a wave-length of 3022-28 Ångström units while that of the Mazda lamps is cut off at about 3100 Ångström units. Our primary object in so far as the quality of the light was concerned, was the absence of rays which are absorbed by ordinary glass, as in the case of the diffused sunlight of the laboratory.

A second room was arranged so that all light was constantly excluded except during the time the animals were fed or examined, when a single 30 watt Mazda lamp was employed. This light was not used for more than 1 hour per day.

An ordinary animal room which has a southern exposure was used as the control room. Practically all the light of this room was diffused sunshine which passed through the glass of two large windows.

The temperature of the three rooms was maintained at 70° to 75°F. by automatic regulation of the common heating and ventilating systems. In the case of the light room, this was supplemented by an electric fan intake of outside air which also served as an aid to satisfactory ventilation. With this equipment, it was not possible to keep the temperature of the light room within the above limits during the warmer months, so that in subsequent experiments to be reported later, the

type of illumination was modified. The humidity of the three rooms varied with that of the outside air.

The rabbits employed were representative of the usual breeds and types commonly used in this laboratory. They were adult male animals, approximately 1 year old, and were assembled from a carefully selected stock 1 week prior to the experiment. During the entire period of observation, each rabbit was separately caged and fed the standard diet of oats, hay and cabbage.

On January 16, 1925, 10 rabbits were placed in each of the three rooms where they remained for the duration of the experiment. On February 16, 1 month after they had been living in an environment of constant light or of constant darkness or in the variable diffused sunlight of the laboratory, each rabbit was inoculated in the right testicle with 0.3 cc. of a tumor emulsion prepared from an actively growing primary tumor. This neoplasm is considered to be of epithelial origin (1) The experiment was terminated on April 15, 2 months after inoculation, at which time all surviving animals were killed by an injection of air into the marginal ear vein. This period was selected upon the basis of previous experience as being sufficiently long to include the majority of deaths due to tumor involvement or to permit recovery to take place.

Detailed records including body weight determinations were kept covering the clinical course of the disease and each animal received a careful postmortem examination with special reference to the presence or absence of metastases, the number and distribution of metastases, the degree of involvement of different organs and the state of the growth. The distribution of metastatic foci in particular has been used as a basis for estimating the general character of the disease in those cases in which secondary growths were found. The method used involves the calculation of percentage values of the number of tumor sites or foci in terms of the number of theoretical foci as shown by the actual location of metastases in the first twenty generations of tumor animals (5). There are certain obvious objections to this method. For instance, such organs as the liver or kidneys may be markedly involved with numerous tumors or by only a few which destroy little tissue. However, a comparative classification of the disease, whether of high, moderate or low malignancy, may be brought out in percentage terms by an arrangement of metastatic distribution according to the following divisions:

I. Skin and subcutaneous tissue, superficial lymph nodes, muscles, heart and pericardium, bones and bone marrow, glands of internal secretion with the exception of the suprarenals, the spleen and the central nervous system...30 possible foci

II. Lungs and pleura, liver, kidneys and pancreas.............5   "   "

III. Extensions and implantations to the retroperitoneal and mediastinal tissues, omentum, mesentery and parietal peritoneum.......................................19   "   "

IV. Suprarenals and eyes................................. 4   "   "

This system of grouping metastatic foci was selected for these reasons:

It has been found, from the study of several hundred rabbits inoculated in the testicle with the tumor, that there is usually a widespread distribution of metastases, extensions and implantations in those animals in which the most malignant disease develops and in which death occurs within 3 to 5 weeks after inoculation. In these instances, tumors may be found in many organs and tissues as in the skin, the superficial lymph nodes, the muscles, the bones and bone marrow, the heart, the spleen, and the endocrine glands as well as in the liver, the kidneys, the lungs, the retroperitoneal and mediastinal tissues and the serous membranes of the abdominal and pleural cavities. In instances of a somewhat less malignant disease, the most conspicuous and frequent metastases are found in the liver, kidneys, lungs and pancreas. A level of still lower malignancy is chiefly characterized by the predominance of extensions of tumor from the primary growth in the testicle to the retroperitoneal and mediastinal tissues and by implantations upon the omentum, mesentery and parietal peritoneum. If death occurs in these animals during the first 2 months after inoculation, the extensions and implantations referred to are found to be of an extreme grade, or, more frequently, some organ such as the kidneys or hypophysis is also involved. Finally, in those animals in which the disease is very mild, metastases may be found only in such sites as the eyes or suprarenal glands which of course are also involved in instances of high malignancy. On the other hand, metastases to the skin, muscles, bones and endocrine gland group practically never occur in cases of low malignancy.

Results.

The results of this experiment consist, first, of the clinical observations in which the general physical condition of the animals, the character of the primary growth and the development of metastases recognizable during life, are of especial interest; second, the mortality rate including the time of death with reference to the time of inoculation; and third, the postmortem observations, particularly as regards the condition of the primary tumor, the animal incidence of metastases and the number, distribution and state of these growths.

As far as could be determined by observation and by body weight, the health of the rabbits kept under the conditions of constant illumination was excellent, and while no outspoken deleterious effects were observed in the animals kept in the dark, there were indications furnished by body weight determinations to the effect that this environment was a less favorable one (Table I). Only the weights of rabbits surviving the experiment have been used in this connection since a loss of weight together with other manifestations of cachexia

frequently occur in association with a widespread tumor growth in animals which succumb to the disease. Reference to the table shows that the groups of rabbits kept in the environment of constant light and of the variable sunlight conditions of an ordinary animal room for 3 months showed a well marked increase in body weight while the group in constant darkness gained comparatively little. Reckoning from the mean weight on admission the controls gained more than the light group due, probably, to the lower initial figure of the controls. However, the final mean weights of both groups were the same. The net gains over the first observations available after the rabbits from the light and dark rooms had been placed in these rooms were practically the same for the light group and the controls, but the dark room group gained only one-third as much as the others.

TABLE I.

Changes in Mean Body Weight.

Group	On admission	Experimental period			Percentage gain	
		1 wk.	4 wks. Day of inoculation	12 wks.	Over admission weight	Over first experimental weight
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
Controls........	2120	2350	2465	2660	25.5	13.2
Lights..........	2264	2342	2428	2675	18.2	14.2
Darks..........	2283	2238	2288	2346	2.7	4.8

A primary tumor developed in each rabbit of the experiment. In the control group,[1] there was a rapid and extensive primary growth for the first 3 weeks, after which time regression took place in the 5 rabbits which survived the experimental period of 2 months, and in 3 of these complete absorption with healing occurred. In the 4 rabbits dying from tumor, there was relatively little necrosis of the primary growth. In the rabbits from the dark room the primary growth was very rapid in 3 animals which early succumbed to the disease, while in 7 rabbits it grew more slowly and ultimately regressed and at the end of the experiment it was entirely necrotic in 2 and

[1] One of the control rabbits died of an intercurrent infection so that this group consists of 9 animals.

healed in 5 instances. In the group exposed to constant illumination, the initial growth of the tumor in the majority of the animals was unusually rapid and extensive but it eventually became entirely necrotic in 5 and completely healed in 4 rabbits. In the remaining animal, which finally succumbed to the disease, approximately one-half of the primary tumor was living.

TABLE II.

Analysis of Results.

Group	No. of rabbits	Metastases found during life		Mortality			Postmortem observations of metastatic foci						Actual and probable recoveries
		Animal incidence	Total No.	No.	Rate	Time after inoculation	Animal incidence	Total No.	Relative rate	Actual rate	No. of foci in		
											Deaths	Survivors	
		per cent			per cent	wks.	per cent						per cent
Controls...	9*	44.4	10	4	44.4	4,4, 4.5, 4.5	56.0	81	9.0	16.2	30, 17, 15, 15	4 in 1 rabbit 0 in 4 rabbits	55.6
Darks......	10	30.0	7	3	30.0	3.5, 3.5, 4	40.0	69	6.9	17.3	26, 25, 17	1 in 1 rabbit 0 in 6 rabbits	70.0
Lights.....	10	20.0	6**	1	10.0	6.5	50.0	27	2.7	5.4	22	5†in 4 rabbits 0 in 5 rabbits	90.0

* 1 rabbit of the original 10 died of an intercurrent bacterial infection during the experiment.

** Two of these metastases eventually became completely healed.

† Three of these metastases occurring in 2 rabbits were small and entirely necrotic.

Metastases in superficial parts of the body which were recognized during life developed in several rabbits (Table II). In the control group there were ten such secondary growths in the eyes, superficial lymph nodes, subcutaneous tissue and bones distributed among 4 rabbits—an animal incidence of 44.4 per cent. In the group kept under conditions of constant darkness, the animal incidence of metas-

tases detected clinically was 30.0 per cent; seven tumors were found in the eyes, lymph nodes, skin and subcutaneous tissue. On the other hand, in only 2 rabbits of the light group—20.0 per cent—were such growths recognized. In 1 rabbit the unusual occurrence of regression and ultimate healing of eye metastases was observed while in another, four tumors were found in the muscles, bones, subcutaneous tissues and superficial lymph nodes.

The mortality rates of the three groups were—controls 44.4 per cent; darks 30.0 per cent; lights 10.0 per cent (Table II). Among the controls, 4 rabbits were killed, 4, $4\frac{1}{2}$ and $5\frac{1}{2}$ weeks after inoculation because of the development of paralysis or other symptoms indicative of impending death. A similar procedure was necessary in three instances in the dark room group, $3\frac{1}{2}$ and 4 weeks after inoculation. Among the rabbits in the light room, on the other hand, there was but one such instance. The symptoms which necessitated the sacrificing of this animal $6\frac{1}{2}$ weeks after inoculation, developed much later and more slowly than those of similar cases in either the control or dark room groups.

At postmortem examination, it was found that the incidence of metastases was approximately the same in all three groups, but there were distinct group differences in the number of foci[2] (Table II). Among the 9 control rabbits there were 81 foci of metastatic growths, or 9 per animal, 69 foci in the dark group, or 6.9 per animal, and only 27 foci in the light group, or 2.7 per animal. A comparison of actual metastatic rates in which only rabbits with secondary growths are considered, shows that there was little difference between the control and dark groups, but in the case of the light group the rate was approximately one-third those of the others.

The number of metastatic foci in individual rabbits is given in Table II. In the fatal cases, the largest number occurred in a con-

[2] It should be pointed out that the figures refer to the number of organs or tissues involved, and not to the numbers of secondary growths, and consequently the expressions "foci of metastases," "distribution of metastases," or "metastatic rate" are used rather than "number of metastases." On the other hand, the figures for clinical metastases detected during the life of the animal refer to the actual number of individual secondary tumors found. The present basis of counting foci of metastases is slightly different from that employed in the preliminary note (4).

trol animal in which 30 foci were counted and in the others, there
were 15 to 26.   But in so far as the severity of the disease may be

Distribution of metastatic foci
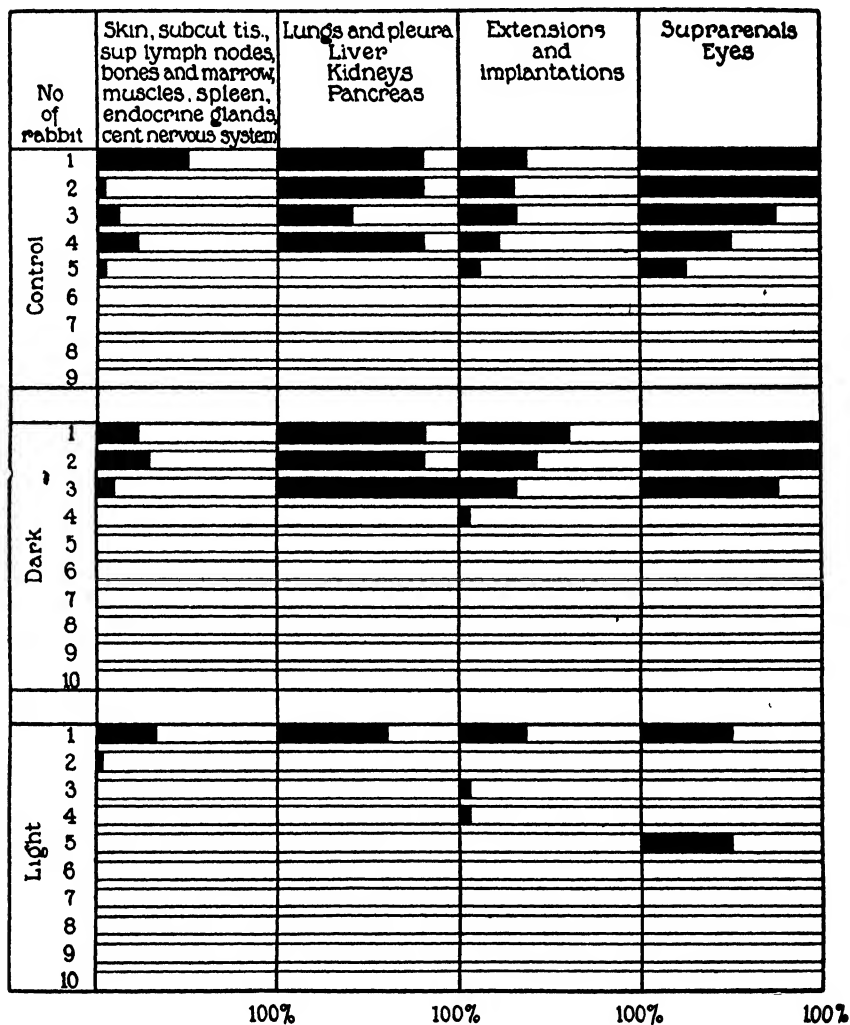
TEXT-FIG. 1. Distribution of metastatic foci.

judged from the mean number of metastatic foci, there was practi-
cally no difference in the three groups—(controls 19.25, darks 22.7,

lights 22.0). Among the rabbits which survived the experimental period of 2 months, postmortem examination revealed 4 metastatic foci in one control, a single focus in a dark room rabbit and 5 foci distributed among 4 light room animals. In 2 of the rabbits from the light room, the three secondary growths found were small and entirely necrotic. It is probable that none of the 6 surviving rabbits with metastatic foci would have died from the effects of tumor growth.

The character of the disease which developed in the three groups may be compared upon still another, and in many respects a more impressive basis, namely, the distribution of metastatic foci, as is illustrated by Text-fig. 1. In this graph, each rabbit is represented by a horizontal column, divided into four sections corresponding to the four general divisions of secondary growths previously described, while the shading of the sections represents the metastatic rate in percentage terms of the possible number of foci.

This diagrammatic representation illustrates in a striking manner the low plane of malignancy of the disease in the light room group. The metastatic rate in all four divisions is seen to be much lower than that of either the controls or the dark room groups. As far as the dark room series is concerned, the metastatic rate is, on the whole, somewhat lower than that of the controls if one takes into consideration the number of animals with secondary growths and the degree of involvement of the two divisions of foci which are predominately affected in instances of severe or well marked malignancy, namely, the division which includes the skin, muscles, bones and glands of internal secretion, and that of the lungs, liver and kidneys. In the case of the light room group, there was but 1 rabbit in which a disease characterized by a widespread distribution of metastatic growths developed while there were 3 such instances among the dark room and 4 among the control animals. In a 5th control rabbit, there was a wider distribution of tumor than in a 4th dark or in 4 other light room rabbits in which metastases were found and in 2 of these last animals, it will be recalled that the secondary growths were entirely necrotic. Finally, there were 4 controls as contrasted with 6 dark and 5 light room animals in which no metastases were found.

DISCUSSION AND CONCLUSIONS.

This experiment was undertaken for the purpose of obtaining information on the influence of light on the character of disease in rabbits induced by a transplantable malignant neoplasm. To this end, the attempt was made to insure conditions which would be common to all animals of the experiment with the exception of the light environment. Comparable conditions of temperature and humidity prevailed in the three rooms in which the experiment was carried out, all the rabbits were fed the same diet, and the animals themselves, assembled at the laboratory at the same time, were well matched as to breed and age. Individual animal variation could not, of course, be wholly eliminated, but this factor was controlled by the selection and number of rabbits employed in each group.

The lighting of the three rooms was the variable factor of the experiment. One group of rabbits, kept in a well lighted animal room was exposed to the daily fluctuations of sunshine diffused through ordinary window glass. Another group was placed in a room which was continuously and constantly lighted by Mazda lamps and mercury arcs in crown glass. The third group was kept in a room from which all light was constantly excluded except for very brief daily periods.

The clinical and postmortem observations of the three groups of rabbits inoculated with the malignant tumor and kept in these different surroundings bring out distinct variations in the course and character of the tumor process. The most highly malignant disease occurred in the control group living under ordinary indoor daylight conditions. The mortality rate was the highest, the incidence of secondary growths detected during life or found at postmortem examination was also the highest and there was the widest distribution of metastatic foci. In contrast to these effects was the extremely mild disease which developed in the rabbits kept under conditions of constant illumination. The mortality rate of this group was only one-fourth that of the controls and the duration of life of the single rabbit which succumbed to the disease was considerably longer than that of comparable controls. In addition, there was a lower incidence and a smaller number of metastases found during life together with a smaller number of metastatic foci observed at autopsy.

The disease which developed in the group living in practically constant darkness was also not as severe as that of the controls but was considerably more malignant than that of the light room group. As compared with the controls, the mortality rate was slightly lower, the incidence and number of clinical metastases were somewhat less, and there were fewer sites of secondary growths as revealed by postmortem examination. In addition, a higher percentage of rabbits either recovered wholly or was in process of recovery. Although the effect of constant light exclusion was much less pronounced than that of constant and continuous illumination, it appeared to be in the same direction of diminished malignancy.

There were certain special features of the disease that developed in the rabbits kept in the light and dark rooms which have a direct bearing on the influence associated with these environments. The course and character of the primary tumor in the control and dark room groups were in general accord with what is usually observed in the majority of normal rabbits. That is, the tumor underwent a rapid growth during the first 2 weeks and, in animals succumbing to the disease 3 to 5 weeks after inoculation, showed little tendency toward regression while in most rabbits surviving as long as 2 months, it was found to be wholly or largely necrotic or completely healed. In contrast with the control and dark room groups, the initial growth of the primary tumor in the majority of the light room rabbits was unusually rapid and extensive so that the first impression of the disease was one of heightened severity. The subsequent course of the primary growth, however, was in agreement with the low level of malignancy shown by the disease of this group, for it became entirely necrotic or became completely healed in all but 1 rabbit.

Although there was practically no difference in the incidence of metastases in the control and light room groups, the greatly lessened number and the markedly restricted distribution of the growths in the rabbits in the light room, indicate that these animals were able to restrain or inhibit the development of the majority of metastatic foci. This finding is further supported first, by the unusual occurrence in 1 rabbit of the complete healing of the metastatic growths in the iris of both eyes; second, by the postmortem finding in 2 other rabbits of but 3 small and entirely necrotic metastatic tumors and

finally by the comparative lateness with which the terminal stages developed in the 1 rabbit of the group which eventually succumbed to the disease.

The incidence of metastases in the dark room group was slightly lower than in the others. The secondary growths were confined almost wholly to 3 rabbits in which the disease exhibited well marked malignant characteristics, while in the other animals it was extremely mild. These results indicate first, that constant darkness has a slight but definite effect upon the course and character of the disease and second, that this effect differs in some respects from that observed under conditions of constant illumination. It would appear that under the influence of the conditions obtaining in the dark room, those rabbits which possessed a high resistance to tumor growth, either natural or acquired, were able to exercise this power more efficiently but that the influence was not sufficient to enable less resistant animals to deal successfully with the disease. On the other hand, the restraining or inhibitive effect associated with constant illumination was, to a greater or less degree, seen in all rabbits of the group.

The results of this experiment are interpreted as furnishing experimental evidence in support of the idea referred to in the beginning of this paper, that there is a relation between the factor of light and the manifestations and outcome of the malignant disease with which we have been working. It will be recalled that, in general, the periods of maximum and minimum sunlight, corresponding with summer and winter were associated with relatively low levels of malignancy while the periods of greatest malignancy occurred at times of abrupt and rapid changes in the hours of sunshine, coinciding roughly with the spring and autumn months.

In the experiment here reported, the most malignant disease developed in the group of rabbits living in an ordinary room, exposed to the influence of the variations in sunlight, passing through ordinary glass, that prevailed during these months (February, March, April). In contrast with these results, a disease of low malignancy occurred in the group kept in an environment of constant and intense illumination. Furthermore, the plane of malignancy of the disease in the rabbits living in a room from which almost all light was excluded was

found to be somewhat lower than that of the controls although it did not approach the low level of the group exposed to constant illumination. It would appear, therefore, that the conditions of light in the experiment reported were associated with variations in the course and character of the malignant disease comparable with those of a seasonal nature which normally occur throughout the year.

The mechanism by which these effects are induced is not known. We have considered the influence of the seasonal factor in disease as operating upon or affecting general animal economy and susceptibility or resistance as a functional activity of the animal organism. Hence it appears that resistance to tumor growth as displayed by rabbits living under conditions of constant intense illumination and, to a less extent, in almost constant darkness is more potent or efficacious than it is in the case of rabbits exposed to the daily fluctuations of diffused sunlight.

Finally, it should be stated that no attempt was made in this experiment to produce ideal conditions for diminishing or enhancing tumor malignancy. The purpose was to determine whether variations in the course and character of the malignant disease took place, first, under conditions of constant and continuous illumination in which none of the shorter ultra-violet rays participated and, second, under conditions of constant darkness.

SUMMARY.

An experiment is reported in which an environment of constant and continuous light excluding the shorter ultra-violet rays, and one of constant darkness, have influenced the course and character of a malignant disease of rabbits induced by a transplantable neoplasm.

Under the influence of constant light the level of malignancy was observed to be low; under the influence of constant darkness the level of malignancy was somewhat lower than in the control animals living under ordinary indoor light conditions, but the level was not as low as among the animals constantly illuminated.

These observations furnish experimental evidence in support of the idea that there is a correlation between the external factor of light on the one hand and the manifestations of an experimental malignant disease on the other.

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THE INFLUENCE OF LIGHT ON THE REACTION TO INFECTION IN EXPERIMENTAL SYPHILIS.

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The occurrence of wide variations in the character and course of the disease produced in rabbits by inoculation with a given strain of *Treponema pallidum* was recognized long since by us as one of the outstanding features of the experimental infection and we have emphasized the fact that the more decided variations show a tendency to a seasonal periodicity with the occurrence of periods of maximum severity at some time during the spring and fall (1, 2). A survey of available data bearing on this subject seemed to indicate a relation between the course of infection and prevailing meteorological conditions (3). This was particularly true of sunlight expressed in terms of the hours of sunshine over a given period of time. But, the data at hand were not sufficient to indicate the precise nature of the relation that obtained or to warrant a definite conclusion as to the relative importance of the several factors that might be concerned in the production of these effects. It seemed, however, that we had to deal with a general rather than a specific problem, or, that the effects noted in the case of syphilis were incidental to the occurrence of profound changes in the animal organism, influenced in some way by physical environment (3).

With a view to obtaining further information on this subject, several lines of investigations were undertaken. One of these was a systematic study of the normal animal organism in relation to physical environment; another assumed the direction of consecutive observations on the course of syphilitic infections in comparable groups of animals over long periods of time, while a third series of investigations was concerned with direct tests of the effects of physical influences on the reaction to infection. Some of the results of these investigations

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have been reported (4, 5). The object of the present paper is to report the results of a series of experiments dealing with the influence of light on the reaction to syphilitic infection. These experiments were undertaken for the purpose of determining, first, whether the course of disease could be influenced by variations in the light environment of animals, comparable to those that occur in consequence of changes in season or weather, and, second, something as to the nature of the effects that might be produced by changes in environment or conditions of this general order. We were, of course, concerned with conditions that might prove to be favorable or unfavorable, but in this instance no effort was made to adjust experimental conditions from this point of view lest by so doing, we might defeat the main object of the experiments. The indications were that at the outset extreme conditions should be employed.

EXPERIMENTAL.

Methods and Material.

The results to be reported represent a comparison of the course of infection in three groups of rabbits living under conditions which, as far as possible, differed only with respect to their light environment. One group of animals, which may be designated as the controls, was kept in a well lighted, well ventilated room with a southern exposure. The temperature of the room was automatically regulated and maintained at a level of 70° to 75°F. while the humidity varied with that of the outside air. The light received by these animals was sunlight which for the most part was filtered through window glass; the amount and quality of the light were determined by natural circumstances.

A second group of rabbits was kept in a room from which all sunlight was excluded; otherwise, the conditions were the same as those of the first room. These animals lived in total darkness except for a brief period each day when it was necessary to use a small Mazda lamp while cleaning cages, feeding, and carrying out routine examinations. For convenience these animals will be referred to as the dark group.

The third group of animals, which may be designated as the light group, was kept in a room similar to the others with sunlight excluded. This room was lighted continuously by 13 Cooper Hewitt, low pressure, mercury arc lights in crown glass (Type P, 50 inches long). The light supplied by these lamps has a wave-length ranging from 3022 to 5790 Ångström units with the greatest concentration between 3650 and 5790. The lamps were mounted on a rectangular frame extending down the middle of the room and were distributed so as to equalize the light which reached cages in a plane parallel with the frame on each side of the

room at a distance of approximately 3½ feet.   With this arrangement of lights, an illumination was maintained which amounted on an average to 200 foot candles, being slightly higher opposite the center of the frame than elsewhere.   To insure proper ventilation and as an aid in controlling the temperature, a special system of ventilation was used in this room.

The conditions compared were, therefore, (1) intermittent and irregular exposure to diffuse, filtered sunlight of variable quality and intensity, (2) complete exclusion of light, and (3) continuous exposure to artificial light of a given quality and intensity.

Three experiments were carried out for the purpose of determining the effects of these conditions on the reaction of rabbits to syphilitic infection.   In each case, 30 male rabbits were used.   They were placed in individual cages and divided into three groups of 10 animals each, as nearly comparable as possible with respect to age, breed, and weight.   The stipulated conditions of the experiments differed in only one respect, namely, the length of time animals were kept under the conditions described prior to inoculation.   In the first experiment, the period was 2 weeks, in the second 4 weeks, and in the third 6 weeks.   There were, in addition, differences referable to conditions that prevailed at the time successive experiments were carried out.   These are of especial interest in connection with the results obtained in control animals.   It is sufficient to say that the first group of animals was inoculated in October, the second in November, and the third in December, 1925.   The period covered by the three experiments was, therefore, from October 1, 1925, to April 9, 1926.

All animals were inoculated in one testicle with the Nichols strain of *Treponema pallidum*.   The emulsions used were prepared from actively growing testicular lesions and contained from 1 to 3 spirochetes to the microscopic field.   Each animal received 0.2 to 0.3 cc. of the emulsion, the exact amount depending upon the spirochetal content.   Animals in a given experiment received equal amounts of the same emulsion and the order of inoculation was the same in all cases, namely, controls, light, dark.

The period of observation following inoculation was 4 months, giving a total period of observation of 4½, 5, and 5½ months respectively for the first, second, and third experiments.

In these experiments, special attention was given to the time and frequency of occurrence and to the duration of successive phases of the reaction to infection with a view to reducing the comparison of results to as accurate a basis of quantitative measurement as possible.   The particular conditions chosen for comparison were, (1) the incubation period of primary lesions, (2) the time and frequency of occurrence of a critical edema in the inoculated testicle, (3) the time and frequency of occurrence of lesions in the uninoculated testicle (metastatic orchitis), (4) the time and frequency of occurrence of generalized lesions in the skin and mucous membranes, bones, or eyes, (5) the number of foci affected by such lesions, and (6) the proportion of animals that showed complete healing of all lesions during the period of observation (4 months).

For the most part, the methods employed in recording results (Tables I and II and Text-figs. 1 and 2) require no explanation. The term "focal distribution" or "focal incidence" as applied to generalized lesions refers to the number of discrete foci at which lesions developed as determined by actual count. The figures for actual distribution are the mean values for those animals of a group that actually developed generalized lesions while the figures for relative distribution give the results in terms of the entire group. This distinction is made in order to permit comparisons of the extent of the lesions irrespective of the number of animals affected and at the same time to avoid any erroneous impression that might arise from the chance occurrence of an occasional case of severe syphilis in any group of animals.

In Table I and Text-figs. 1 and 2 the results for focal distribution are given in absolute numbers; all other results are in per cent.

Results.

The results of the experiments are recorded in Tables I and II, and Text-figs. 1 and 2. It will be noted that the figures given in Tables I and II represent group values; the detailed results are given in Text-fig. 1 which shows the entire sequence of events from the occurrence of the first primary lesion to the development of the last generalized lesion.

TABLE I.

Incidence of Various Phenomena of Infection and Focal Distribution of Generalized Lesions Actual (F. D. Act.) and Relative (F. D. Rel.).

Exp.		Primary lesions	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
					Incidence	Focal distribution	Focal distribution
		per cent	*per cent*	*per cent*	*per cent*	*actual*	*relative*
I	C.	100.0	40.0	100.0	80.0	8.0	6.4
	D.	100.0	60.0	100.0	100.0	5.3	5.3
	L.	100.0	90.0	80.0	70.0	3.6	2.5
II	C.	100.0	30.0	100.0	70.0	4.1	2.9
	D.	100.0	70.0	90.0	80.0	5.0	4.0
	L.*	100.0	77.7	88.8	77.7	2.3	1.6
III	C.	100.0	40.0	100.0	70.0	4.1	2.9
	D.	100.0	70.0	100.0	100.0	5.3	5.3
	L.	100.0	80.0	70.0	50.0	4.8	2.4
Mean values	C.	100.0	36.6	100.0	73.3	5.4	4.1
	D.	100.0	66.6	96.6	93.3	5.2	4.9
	L.	100.0	82.5	79.6	65.9	3.6	2.2

* One animal in this group died at the beginning of the experiment; there was also 1 death in the control group and 1 in the dark at the termination of the first experiment.

TABLE II.

Mean Time of Occurrence of Various Phenomena of Infection Estimated in Days from the Date of Inoculation.

Exp.		Incubation	Edema	Metastatic orchitis	Generalized lesions		
					First	Last	Mean of all
		days	*days*	*days*	*days*	*days*	*days*
I	C.	12.6	21.5	41.0	54.9	82.4	65.5
	D.	12.4	26.6	42.7	53.9	76.4	60.4
	L.	13.0	27.2	36.4	58.8	73.4	65.2
II	C.	16.1	33.7	45.8	55.0	70.3	69.4
	D.	12.8	30.0	46.6	59.5	84.1	67.1
	L.	14.4	34.6	46.3	63.9	66.4	63.1
III	C.	18.1	28.8	39.6	63.7	72.4	65.0
	D.	11.3	29.3	45.3	53.1	72.4	61.1
	L.	15.2	25.4	50.1	56.2	65.0	61.1
Mean values	C.	15.6	28.0	42.1	57.9	75.0	66.6
	D.	12.2	28.6	44.9	55.5	77.6	62.9
	L.	14.2	29.1	44.2	59.6	68.3	63.1

TEXT-FIG. 1.

Incidence and focal distribution of generalized lesions
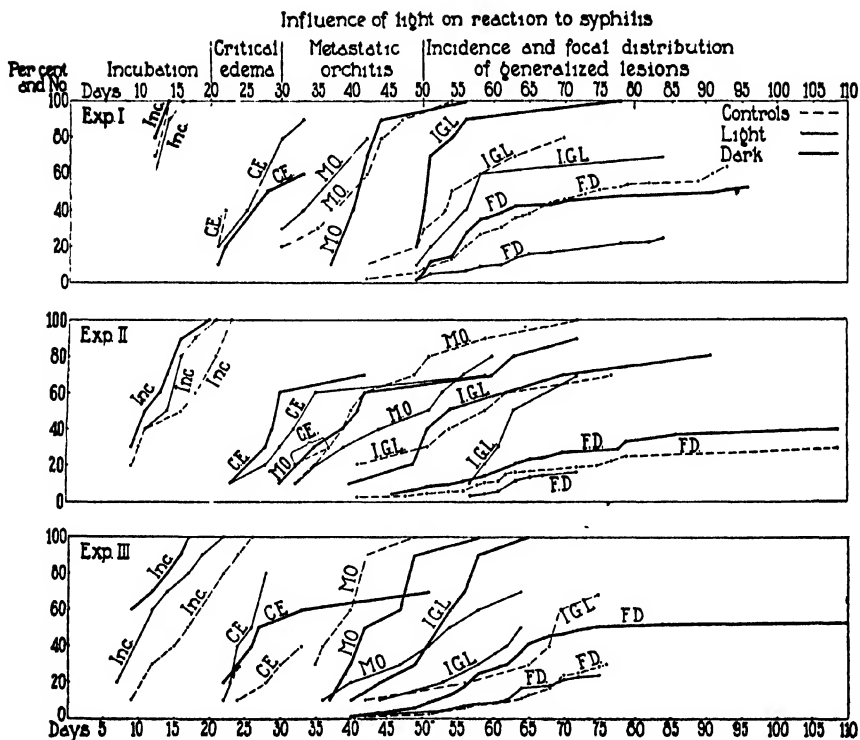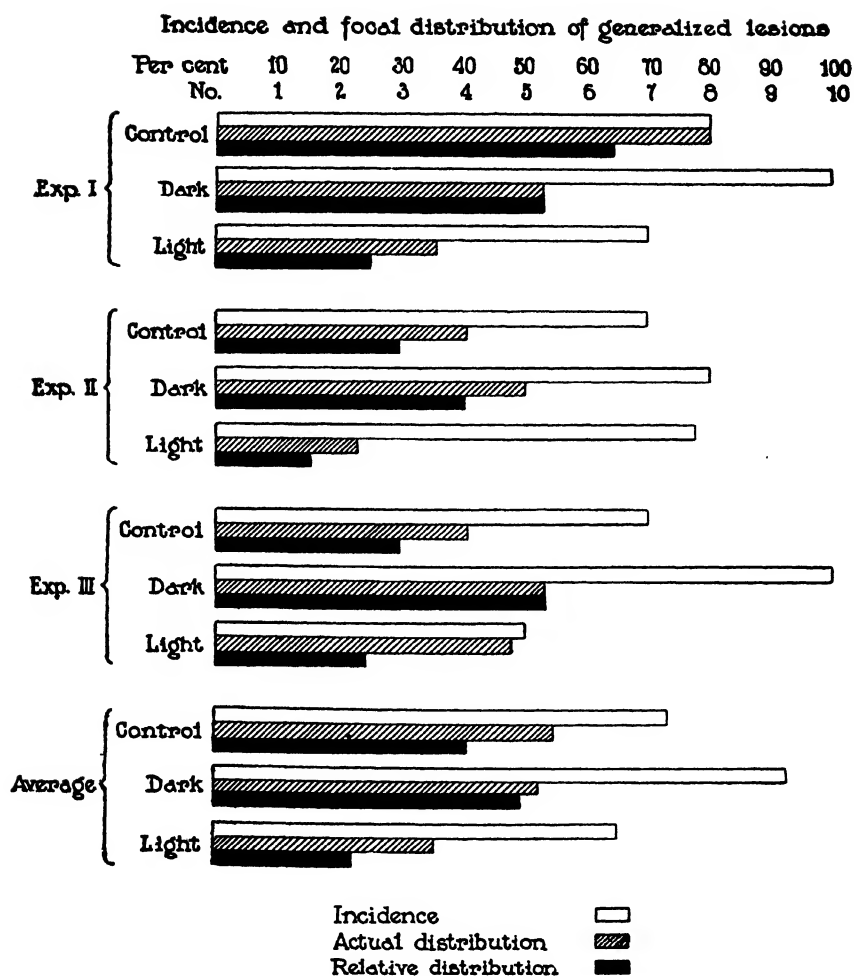
TEXT-FIG. 2.

DISCUSSION AND CONCLUSIONS.

In analyzing the results of the experiments, the most important point to be determined is whether the light environment of the three groups of animals produced any significant difference in their reaction to infection. The effect of any particular condition on the course of disease is of subsidiary importance. The simplest method of approaching this problem is by a comparison of the results obtained for various reactive phenomena in the order of their occurrence, bearing in mind the relations that obtain and the general principles that govern the evolution of syphilitic infections (6).[1] Moreover, the discussion may be limited almost entirely to a consideration of values obtained for group means as the results given by a more detailed analysis of the behavior of individual animals or a consideration of the character of the lesions produced lead to exactly the same conclusions.

Incidence of Primary Lesions and Length of Incubation Period.—All animals in these experiments developed primary lesions but there were some suggestive differences in the incubation period. In the first experiment (Table II, and Text-fig. 1), the incubation period was essentially the same for all three groups of animals with the controls occupying a position intermediate between those in the light and those in the dark. In the second and third experiments, the controls showed a progressive prolongation of the incubation period which amounted to approximately 40 per cent. The animals exposed to artificial light displayed a similar tendency but the differences were less. In the case of the animals in the dark, the incubation period was remarkably constant and such differences as occurred were in the direction of a reduction rather than a prolongation of the time. Combined results for the three experiments, with an average of 15.6 days for the controls, 14.2 for the light, and 12.2 days for the dark group, give a fair index of the promptness of the primary reaction to infection in the three groups of animals.

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[1] In addition to the relations that are discussed in the paper referred to (6), it should be noted that there is a basic tendency to the preservation of a uniform interval of time between the occurrence of successive reactions in syphilitic rabbits. With highly virulent strains of *Treponema pallidum* the interval, or reaction time, is approximately 2 weeks and any decided departure from this basic value may be regarded as significant.

Critical Edema.—The occurrence of a critical edema in an inoculated testicle or in association with any other syphilitic lesion is a very variable but highly significant phenomenon. It is indicative of an intense reaction and usually marks the termination of a local reaction, either temporary or final (7).

In the present instance, there was a very striking and constant difference in the frequency with which edema occurred in the three groups of rabbits. The differences are well represented by the combined results for the three experiments which give 36.6, 66.6, and 82.5 per cent respectively for the control, dark, and light groups.

The figures given in Table II indicate a closer agreement with respect to the time at which the edema occurred. In the case of both the control and light groups, the interval between inoculation and the development of edema varied considerably from one experiment to another but remained fairly constant for animals in the dark. If, for the purpose of measuring the progress of the reaction, the time, in days, is estimated from the appearance of the primary lesions, instead of the date of inoculation, the results are slightly different:

	Control	Dark	Light
Exp. I.................	8.9	14.2	14.2
" II.................	17.6	17.2	20.2
" III.................	10.7	18.0	10.2
Mean.................	12.4	16.5	14.9

Still, these figures, as well as those given in Table II, are somewhat misleading. By reference to Text-fig. 1, it will be seen that, with the the exception of 1 animal each in the light and dark groups of the second experiment and 1 in the dark group of the third experiment, the time of occurrence of edema in individual animals was fairly uniform. The delay in the occurrence of the reaction in the 3 animals referred to overshadows the group tendency so that if the figures are corrected by the omission of these animals, we obtain the following results:

From Inoculation.

	Control	Dark	Light
Exp. I.....................	21.5	26.6	27.2
" II.....................	33.7	26.0	28.5
" III.....................	28.8	25.5	25.4
Mean....................	28.0	26.0	27.0

From Incubation.

	Control	Dark	Light
Exp. I.....................	8.9	14.2	14.2
" II.....................	17.6	13.2	14.1
" III.....................	10.7	14.2	10.3
Mean....................	12.4	13.9	12.9

A comparison of results on this basis shows a remarkable constancy in the occurrence of edema among the animals in the light, as well as those in the dark, with a possible tendency toward a shortening of the reaction time. The differences in mean time for the three experiments are comparatively slight, but they are of interest as measures of the progress of the reaction to infection. For example, it will be noted that the time from inoculation to the occurrence of edema in the animals in the dark was shorter than in the controls or the light group, due largely to differences in the incubation period, whereas the time from the development of primary lesions was longer. These differences are suggestive of differences in the efficiency of the reactive mechanism which become more apparent in subsequent reactions.

The most important points brought out by the analysis of the results with respect to the occurrence of a critical edema, are, therefore, the high incidence of edema and the constant time relations shown by both the animals in the dark and those in the light as compared with the much lower incidence and irregular reaction time of the controls.

Metastatic Orchitis.—The development of lesions in an uninoculated testicle occurs with such regularity in rabbits infected with virulent strains of *Treponema pallidum* that the failure of such lesions to occur

may be regarded as evidence of an unusually effective suppression of the infection by some preceding reaction. Variations in the time of development of metastatic lesions are apt to occur and in general the time of occurrence bears a direct relation to the efficiency of preceding reactions.

The outstanding feature of the results as regards the occurrence of metastatic orchitis is the diminished frequency of such lesions in the light animals as compared with the two other groups (Table I). This variation from the expected course occurred in all three experiments but was most decided among those animals that had been exposed to artificial light for the longest time.

The time relations of the reaction are more difficult to interpret. The mean values for the three experiments (Table II) show a comparatively close agreement despite the differences obtained in the first and third experiments and the very irregular development of lesions shown by all groups of animals in the second experiment (see Text-fig. 1).

The situation is clarified somewhat by comparing time intervals (in days) from the development of primary to metastatic lesions and from the occurrence of edema to metastatic orchitis as follows:

From Development of Primary Lesions.

	Control	Dark	Light
Exp. I....................	28.4	30.3	23.4
" II....................	29.7	33.8	31.9
" III....................	21.5	34.0	34.9.
Mean....................	26.5	32.7	30.1

From Occurrence of Edema.

	Control	Dark	Light
Exp. I....................	19.5	16.1	9.2
" II....................	12.1	16.6	11.7
" III....................	10.8	16.0	24.7
Mean....................	14.1	16.2	15.2

This analysis shows that as we narrow down our basis of reckoning from the rather remote event of inoculation to the occurrence of the reaction immediately preceding the development of metastatic orchitis, relations that are more or less obscure become clearly defined. It is at once apparent that conditions that determined the time of development of metastatic orchitis were not the same in any two groups of animals. The light group and the controls are diametrically opposed whereas the animals in the dark maintain a constant relation from one experiment to another just as they did in the development of primary lesions and edema. In view of what has been said with respect to the significance of the time relations of the reaction, it is important to note that the animals exposed to artificial light showed a progressive prolongation of the reaction time.

Generalized Lesions.—The occurrence or non-occurrence of generalized lesions, the character and extent of the lesions, the time of their appearance, the duration of the period of active development, and the persistence of the lesions afford the most acceptable basis for estimating the effectiveness of the reaction to syphilitic infection in the rabbit. As is well known, the course of disease is subject to wide variation in all of these respects, but, as a rule, is comparatively constant under a given set of conditions.

With respect to the incidence of generalized syphilis, the figures given in Table I show that the condition was definitely more frequent among the animals in the dark and slightly less frequent among those in the light than among the controls. It will be noted, however, that there were fewer cases of generalized syphilis among the control animals of the second and third experiments than in the first and a marked reduction in the number of foci affected (focal distribution, Table I). The animals in the light also showed a reduction in the incidence and distribution of lesions which followed a slightly different order but those in the dark showed very little variation in either of these respects. The relations that obtained are shown graphically in Text-fig. 2.

The apparent reduction in the incidence and distribution figures for the dark group of animals in the second experiment is probably due to a change in the character of the lesions. In the first experiment, the majority of lesions in all three groups of animals were comparatively large and of a destructive character. This was especially

true of controls and of animals in the dark, and in both of these groups there were several cases of unusually severe generalized syphilis. Among the light animals, however, the lesions were smaller, less destructive, and of shorter duration.

There was no material change in the character of the lesions presented by animals of the control and light groups in the second and third experiments. Among the animals in the dark, however, the character of the disease changed. The first lesions tended to be small and of short duration, but were prone to relapse and some of the recurrent lesions attained a large size. This peculiarity of the reaction was at first confusing and, in the second experiment, it was not until some of the lesions had recurred a second or perhaps a third time that the significance of the condition was fully appreciated and a definite diagnosis of generalized syphilis was made. It is probable, therefore, that the incidence and distribution figures for these animals in the second experiment are too low and it is certain that the time given for the occurrence of the first generalized lesions is too long; hence, some allowance should be made for this error in the calculations which involve the time of occurrence of these lesions. The values given for the third experiment are little if at all affected by this factor.

The time relations of the reaction to generalized infection may be viewed in a number of ways. The figures given in Table II show no striking difference in the time from inoculation to the development of generalized lesions in the three groups of animals. The relations change from one experiment to another, but one is impressed chiefly by the closeness of the agreement and the narrow limits of variation. The mean results for the three experiments suggest a precocious development of lesions among the animals in the dark and perhaps a retarded development in the light group if we consider the time at which the first generalized lesions appeared, that is the onset of the generalized reaction, as shown by the following tabulation:

	Control	Dark	Light
Exp. I....................	54.9	53.9	58.8
" II....................	55.0	59.5	63.9
" III....................	63.7	53.1	56.2
Mean....................	57.9	55.5	59.6

If we take the mean time of development of all generalized lesions, the situation is slightly different.   The results in this case are as follows:

	Control	Dark	Light
Exp. I.....................	65.5	60.4	65.2
" II.....................	69.4	67.1	63.1
" III.....................	65.0	61.1	61.1
Mean.....................	66.6	62.9	63.1

Considered from this point of view there is a suggestion that generalized lesions developed earlier on the whole in both the dark and light groups than in the controls.

The results as to the duration of the period of active secondary eruption are more conclusive.   The mean time of appearance of the last lesions in each animal was as follows:

	Control	Dark	Light
Exp. I.....................	82.4	76.4	73.4
" II.....................	70.3	84.1	66.4
" III.....................	72.4	72.4	65.0
Mean.....................	75.0	77.6	68.3

This definitely indicates an earlier termination of the period of secondary reaction in the animals of the light group and a probable prolongation of the period in the dark group.   Greater differences are shown, however, by a comparison of the mean time between the development of the first and the last lesions in all animals of the three groups as indicated below:

	Control	Dark	Light
Exp. I.....................	27.5	22.5	14.6
" II.....................	15.3	24.6	2.5
" III.....................	8.7	19.3	8.8
Mean.....................	17.2	22.1	8.6

These figures show not only a difference in the character of the reaction, but they furnish an excellent index of the efficiency of the reaction developed by the three groups of animals.

There is one other comparison that may be made for the purpose of bringing out an important relation between the occurrence of generalized lesions and metastatic orchitis. The time interval between these reactions may be estimated from the appearance of the first generalized lesions or from the mean time for all lesions. The respective results are:

From Metastatic Orchitis to Time of First Generalized Lesions.

	Control	Dark	Light
Exp. I......................	13.9	11.2	22.4
" II......................	9.2	12.9	17.6
" III......................	24.1	7.8	6.1
Mean......................	15.7	10.6	15.4

From Metastatic Orchitis to Mean Time for All Generalized Lesions.

	Control	Dark	Light
Exp. I......................	24.5	17.7	28.8
" II......................	23.6	20.5	16.8
" III......................	25.4	15.8	11.0
Mean......................	24.5	18.0	18.9

The results obtained by these comparisons show that, while in successive experiments the time between the occurrence of metastatic orchitis and the initiation of the reaction to generalized infection in the control animals was irregular, the mean time between the two reactions was virtually constant. This was not true of either of the experimental groups. The initial time between the two reactions was affected in precisely the same manner and, while from one experiment to another the animals in the dark displayed less variation than those in the light, both showed a progressive reduction of the reaction time. That is, the longer the animals had been exposed to a given condition, the shorter the interval between the development of metastatic orchitis and generalized lesions.

These results are of interest on account of their bearing on the question of the efficiency of the reaction displayed. In the case of animals in the dark, the significance of the results is at once apparent. The short interval between the development of the two sets of lesions was associated with a high incidence of metastatic orchitis and generalized lesions and is to be regarded, therefore, as evidence of an ineffectual reaction. But, the occurrence of a similar reduction in the reaction time of the light animals was associated with two unusual conditions, namely, absence of infection in the uninoculated testicle of some animals and delayed development of lesions in others; in addition, the incidence of generalized lesions was low. It is evident, therefore, that the efficiency of the reaction displayed by these animals was of a higher order, in fact, sufficiently potent to disturb the usual course of events to such an extent as to cause a gradual overlapping of metastatic orchitis and generalized lesions (see Text-fig. 1) but not sufficient to entirely prevent the occurrence of either of these conditions. In the end, generalized lesions actually increased in proportion to the suppression of the reaction in the uninoculated testicle due undoubtedly to the failure of the protection usually afforded by such reactions, hence, the reaction time in the light animals bears an inverse relation to the suppression of lesions in the uninoculated testicle or to the inhibitory effect of the reaction in the inoculated testicle.

This result is not uncommon with strains of *pallidum* of low virulence but is very unusual with strains of high virulence, as under ordinary circumstances, the reaction in the inoculated testicle is rarely sufficient to prevent or materially delay the occurrence of metastatic orchitis. The infection progresses virtually unimpeded until lesions develop in both testicles and not until then does the reaction become sufficiently potent to prevent the occurrence of other lesions. It is evident, therefore, that the primary reaction in the light animals was unusually effective and the same may be said of the secondary reaction as the incidence of generalized lesions was comparatively low and the duration of the period of active development was unusually short.

Recovery.—A final comparison of the behavior of the three groups of animals may be made on the basis of the proportion of animals of each group that showed complete resolution and healing of all lesions

within the period of 4 months' observation. The results, expressed in per cent, were as follows:

	Control	Dark	Light
Exp. I.....................	30.0	50.0	70.0
" II.....................	10.0	50.0	40.0
" III.....................	50.0	40.0	30.0
Mean....................	30.0	46.6	46.6

Here, again, we find that the results for the controls are irregular while those for the animals in the dark are fairly uniform and the light animals show a progressive reduction in the percentage of recoveries with mean values for both the light and dark groups that are higher than those for the controls. The peculiar feature of these results is the diminishing percentage of recoveries among the light animals which is attributable to a combination of circumstances analogous to those that were pointed out in the discussion of the relations between metastatic orchitis and generalized lesions; that is, an early partial suppression of the infection with consequent delay in the development of full immunity.

Some additional evidence as to the influence of the light environment may be adduced from the records of weight which give a fair index of the physical condition of the three groups of animals during the period covered by the experiments. These results may be tabulated in the form of percentage gains in weight from the beginning to the conclusion of the experiments.

	Control	Dark	Light
Exp. I.....................	21.4	33.5	39.8
" II.....................	38.1	25.6	53.9
" III.....................	31.2	34.1	43.3
Mean....................	30.2	31.1	45.7

These results are of essentially the same order as those obtained for various phases of the reaction to infection. In fact, the resemblance is so close that one can hardly escape the conclusion that the two sets of results represent closely related effects of a common cause.

The interpretation of results will be facilitated by calling attention to one other feature of the experiments, namely, the differences in the severity of the disease shown by control animals of the three experiments in relation to prevailing conditions of sunlight.   By reference to tables and text-figures, it will be seen that, in the first experiment, the infection was much more severe than in the second or third.   The comparative results for incidence and distribution of generalized lesions were:

	Incidence	Actual distribution	Relative distribution
Exp. I...................	80.0	8.0	6.4
" II...................	70.0	4.1	2.9
" III...................	70.0	4.1	2.9

The monthly hours of sunshine (Central Park Observatory, New York) for the period immediately preceding and covered by the experiments were:

	Mean normal	Actual	Departure from normal
August.....................	272.3	316.0	+43.7
September..................	230.4	210.4 .	−20.0
October...................	200.8	193.3·	−7.5
November..................	152.6	184.0	+31.4
December..................	140.2	168.2	+28.0
January...................	139.7	161.8	+22.1
February..................	158.5	164.3	+5.8
March.....................	203.7	274.2	+70.5
April.....................	227.7	280.9	+53.2

The points of interest brought out by these figures are, first, the marked reduction in sunshine immediately preceding and following the inoculation of the first group of animals (Experiment I), second, the (unusually) high value for November, and, third, the maintenance of an almost constant and unusually high level of sunshine for the months of December, January, and February which covers the critical period of the second and third experiments.   As will be seen, the actual level of sunshine for the 3 months was slightly higher than the normal November and February level or represented a mid-fall and early spring rather than a winter condition.

Without attempting to analyze these relations in detail, it may be pointed out that the occurrence of the most severe infection followed an abrupt and decided change in the prevailing level of sunshine and that, with the stabilization of the sunshine curve at a slightly lower level, the infection assumed a milder form; there were irregularities in the progress of the disease, just as the hours of sunshine varied from week to week, but the final results for the second and third experiments were essentially the same. These facts have a direct bearing on the results obtained in other groups of animals and are of especial interest in connection with effects produced by constant exposure to artificial light.

In attempting to arrive at a conclusion concerning the significance of the results reported, the distinctive features of the reaction displayed by each group of animals should be viewed in relation to the nature of the experimental condition represented. The variable factor in the experiments was the light environment. Other factors were either constant or the variations that occurred were common to the three groups of animals. For reasons that have been explained, the conditions compared were extreme; with one group of animals, light was excluded so that if it were capable of influencing the reaction to infection, this influence would be completely eliminated. The course of disease in these animals was characterized first by uniformity or constancy with respect to both the incidence and the time of occurrence of successive reactions or lesions and second, by a high incidence of generalized syphilis of a severe character. That is, the infection pursued a uniform course from inoculation to the development of outspoken secondary syphilis with comparatively little restraint during the early stages of infection.

A second group of animals was provided with a constant and continuous supply of artificial light of high intensity with a view to affording an opportunity for the development of any effect which the light might be capable of producing, free from any possible disturbing influence of periodic variation. In this case, the severity of the disease diminished with successive experiments as the length of exposure to light increased. There was an increased intensity of primary reactions with a progressive delay and diminished frequency in the occurrence of secondary reactions including metastatic orchitis and generalized

lesions.   The picture presented suggested an accentuation of primary reactions with increased effectiveness and consequent delay or prevention of other lesions—a condition almost diametrically opposed to that presented by the animals from which all light was excluded.

It should be borne in mind, however, that placing the animals in darkened or artificially lighted rooms entailed a change in the light environment to which they were accustomed.   This may have affected the results of the first experiment in particular and affords a possible explanation for the fact that, among the dark animals, the disease attained its maximum severity in the first experiment, was somewhat milder in the second, and again more severe in the third experiment.

The condition represented by the control animals differed from that of the animals receiving artificial light in three essential respects, namely, the character of the light, and the intensity and constancy of illumination.   The results obtained differed also with respect to the general character of the reaction to infection, the relative frequency of occurrence of various reactive phenomena, and especially with respect to the regularity in the evolution of the disease from one experiment to another; but, the final results showed an evident tendency in the same general direction.

On the whole, it may be said that the results tended to conform to the nature of the experimental condition represented.   In the control animals, the reaction to infection varied in accordance with the conditions under which the animals lived; the animals provided with artificial light showed a progressive increase in the efficiency of the reaction corresponding with the length of exposure to the light while among those in the dark there was a gradual lowering of the efficiency of the reaction to infection, if we measure efficiency in terms of prevention or delay in the development of successive manifestations of disease.

It is obvious that these effects are attributable to the conditions under which the animals lived rather than to any condition inherent in the material used for inoculation or to chance variation.   It is also evident that the course of disease in the three groups of animals may have been influenced in a common direction by a number of unknown factors, including the material inoculated and there is unmistakable evidence of the action of such factors.   The differences shown, how-

ever, can be accounted for only on the basis of differences in the light environment, the effect of which may have been accentuated or diminished by the influence of other factors. Moreover, it is apparent that the reaction of animals may be influenced by conditions to which they are exposed either before or after the initiation of the infection.

The mechanism concerned in the production of these effects is unknown, but since it has been shown that the light environment influences the physical constitution of normal rabbits (5) and that the organs most affected are organs that are concerned in the reaction to syphilitic infection (2, 8), it is logical to assume that the effects produced in this case represent expressions of functional activity which are closely related to changes in physical constitution.

Finally, the greater efficiency of the reaction displayed by animals exposed to artificial light as compared with those living in darkness and the maintenance of a better physical condition speak for a beneficial influence of light which is supported by the results obtained in control animals. There are, however, no grounds for assuming that the optimum effect was attained by the particular conditions that were employed in these experiments.

A comparison of effects due to filtered sunlight and to artificial light *per se* cannot be made as the condition complex represented by one form of light was variable and uncontrolled while that of the other was constant. The superiority shown by the artificial light may have been referable to the constancy of exposure rather than to differences in either the quality or the intensity of the light. It is evident that changing conditions of light environment are a disturbing factor and that in extreme cases the occurrence of such changes may completely counterbalance any beneficial effects of the light itself; but, the relative importance of the several factors that determine the effects of light on the animal organism and the general scope of their action are still uncertain.

SUMMARY.

A series of experiments was carried out for the purpose of determining whether the reaction of rabbits inoculated with *Treponema pallidum* might be influenced by their light environment. The condi-

tions compared were (1) diffuse sunlight filtered through window glass and subject to variations due to natural causes, (2) constant and continuous exposure to artificial light with a wave-length of from 3022 to 5790 Ångström units (Cooper Hewitt), and (3) complete exclusion of light.

The results showed clearly that each of these conditions produced a distinctive effect and that the effect tended to conform to the nature of the environmental condition. In general, the efficiency of the reaction to infection increased with the amount of light received and with the constancy of the exposure.

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EPIDEMIOLOGICAL STUDIES ON RESPIRATORY INFECTIONS OF THE RABBIT.

IX. THE SPREAD OF BACTERIUM LEPISEPTICUM INFECTION AT A RABBIT FARM IN NEW CITY, N. Y.

AN EPIDEMIOLOGICAL STUDY.

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A comprehensive experimental study of the mode of spread of a bacterial disease demands knowledge of its natural epidemiology (1, *a*). This includes data describing the natural prevalence and distribution of the inciting organism, the frequency of various bacterial types in nature, their normal portal of entry into the body of the host, the different responses of the host to the microbe, and the frequency of each response under varying hereditary and environmental conditions. Such information regarding the natural prevalence of an animal disease has at least two important uses in experimental epidemiology,—first, it enables one to induce the infection experimentally, under conditions which simulate nature closely, thereby reducing the number of disturbing artifacts; second, it serves as a control with which to evaluate the conclusions derived from the precise, yet necessarily limited experiments of the laboratory.

As an aid to our experimental studies of *Bact. lepisepticum* infection of rabbits, we have observed the native disease as it occurs among the stock at The Rockefeller Institute and at a rabbit farm at New City, N. Y. The findings in the former group, during the years 1923 and 1924, may be reviewed briefly as follows.

Bact. lepisepticum infection has been found present in about 60 per cent of the rabbits at The Rockefeller Institute (1, *b*). From 20 to 40 per cent of the unused, "normal" stock showed clinical or pathological evidence of snuffles, 5 per cent

evidence of otitis media (1, *c*), 30 per cent were "healthy," nasal carriers (1, *d*), and 20 per cent appeared normal and were not carriers. Among the animals subjected to operation, or otherwise used for experimental purposes, the percentage of infection was greater—in one such series, 40 per cent showed evidence of snuffles as compared to 20 per cent in the corresponding control group (1, *e*); in another, otitis media, 45 per cent, as compared to 5 per cent in unused stock (1, *c*). Inoculations of injurious substances were found to increase the incidence of the "spontaneous" infection by as much as 50 per cent (1, *e*). Both "D" and mucoid types of *Bact. lepisepticum* were encountered at this time (1, *f*).

The New City group of rabbits was studied in a somewhat similar manner and the findings are described in the present paper.*

Source of Material.

The present studies were made at a commercial rabbitry in New City, N. Y.[1] The farm is situated in open country, 200 feet above sea level, in a sparsely populated community. The buildings are placed on a tract of land comprising several acres and are isolated by means of a surrounding wire fence, 8 feet high. A number of watch dogs serve as further protection from animal marauders. Three buildings were in use during the first months of our observations; later, a fourth was added. In general architecture, they are all similar (Text-fig. 1). They measure 100 feet by 20 feet, with the long dimensions facing east and west. Each building contains two rows of wooden cages, running the length of the structure, arranged in tiers of four from ceiling to floor. About 368 cages, measuring 5 feet by 2.1 feet by 1.6 feet each, are contained in each building. The cages consist of a front runway facing toward the open east or west exposure, and a rear partition, kept relatively dark and used for breeding. Each house is cared for by two men who maintain a uniform and efficient system of cage cleaning and feeding throughout. Temperature fluctuates during the year according to outside weather conditions. No artificial heat is used. The side doors, east and west, are thrown open whenever possible, thus making conditions practically "out-of-doors." Food consists of hay, a grain mixture of oats, oatmeal, and wheat, fresh carrots, cabbage, and greens, according to the season. During the winter cold storage carrots are used.

The rabbit population is composed almost entirely of pedigreed and registered stock About 50 per cent are Chinchillas, 15 per cent Blue Beverens, 10 per cent Havanas, 10 per cent Belgian hare crosses with Chinchillas, and 15 per cent Gaudas, Lilacs, and Angoras. Pure line breeds were thus available for comparative study.

The animals are distributed as follows: Building 2, all breeds; No. 3, Chinchillas; No. 4, Chinchillas, Blue Beverens, and Angoras; No. 6, Chinchillas. Each rabbit 6 months old or over occupies a separate cage. The females are bred four or five times a year; four males are used for twenty females. Complete breeding records are kept. The young remain in the cage with the mother until they are old enough to be placed in individual cages.

TEXT-FIG. 1. Building 6, showing open side doors and arrangement of individual rabbit pens. Photograph by Edwin Levick furnished by Mr. Haack.

Methods of Study.

The spread of *Bact. lepisepticum* infection among this group of rabbits was studied as follows. Animals 6 months of age or over, occupying individual cages, were selected for observation. The number of these varied from 841 to 1325. The young were disregarded

(1) From November, 1924, until November, 1926, complete mortality records were kept. (2) From November, 1924, until April, 1925, every rabbit that died was wrapped in paper, placed on ice, and sent to The Rockefeller Institute, where a complete autopsy was performed and cultures were taken. (3) During November, 1924, February, May, and August, 1925, a clinical examination of each rabbit was made. Its general condition was noted, signs of *Bact. lepisepticum* infection were searched for carefully, and other abnormalities were recorded. (4) Finally, a group of clinically normal Chinchillas and Blue Beverens was tested for fre-

TABLE I.

Summary of Data on the Mortality and Incidence of Snuffles among the Rabbits at the New City Farm, N. Y.

Date of observation	Total No. of rabbits examined	Total per cent snuffles	Total mortality per 1000	Bact. lepisepticum infection mortality per 1000	Intestinal infection mortality per 1000	Miscellaneous causes mortality per 1000	Chinchilla breed Total No.	Mortality No.	Mortality Per cent	Snuffles No.	Snuffles Per cent	Havana breed Total No.	Mortality No.	Per cent	Snuffles No.	Per cent	Blue Beveren breed Total No.	Mortality No.	Per cent	Snuffles No.	Per cent
1924																					
November	1016	46.2	30.5	14.75	8.86	6.9	576	19	3.3	285	49.4	117	5	4.2	50	42.7	82	1	1.2	20	24.3
December	—	—	*48.3	23.6	16.75	7.9	—	27	4.7	—	—	—	8	6.8	—	—	—	4	4.8	—	—
1925																					
January	—	—	*40.3	24.6	12.8	2.95	—	31	5.3	—	—	—	9	7.7	—	—	—	1	1.2	—	—
February	1015	48.9	27.6	12.8	10.9	3.94	503	19	3.8	268	53.2	118	4	3.4	64	54.2	112	1	0.9	39	34.8
March	—	—	*47.4	27.6	11.8	7.9	—	26	5.1	—	—	—	7	5.9	—	—	—	2	1.8	—	—
April	—	—	*75.9	—	—	—	—	40	7.9	—	—	—	15	12.7	—	—	—	3	2.6	—	—
May	1116	49.1	106.7	—	—	—	528	64	12.1	287	54.3	118	18	15.3	68	57.6	196	11	5.6	54	27.5
June	—	—	*89.5	—	—	—	—	57	10.8	—	—	—	11	9.3	—	—	—	4	2.0	—	—
July	—	—	*28.7	—	—	·	—	20	3.8	—	—	—	4	3.4	—	—	—	1	0.5	—	—
August	1308	43.3	28.1	—	—	—	564	23	4.0	284	50.3	117	6	5.2	78	66.6	214	1	0.3	68	24.1
September	—	—	*31.2	—	—	—	—	17	3.0	—	—	—	4	3.4	—	—	325	8	2.5	—	—
October	1325	—	28.7	—	—	—	630	17	2.7	—	—	—	4	3.4	—	—	350	5	1.4	—	—
November	1310	—	24.4	—	—	—	620	10	1.6	—	—	—					350	6	1.7	—	—
December	1113	—	39.5	—	—	—	550	18	3.3	—	—	—					300	14	4.7	—	—
1926																					
January	1087	—	37.8	—	—	—	550	22	4.0	—	—	—					285	9	3.20	—	—
February	971	—	36.0	—	—	—	490	16	3.3	—	—	—					260	5	1.9	—	—

March	954	—	24.0	—	—	490	14	2.9	—	230	4	1.7	—	—
April	1016	—	33.5	—	—	490	22	4.5	—	250	6	2.4	—	—
May	961	—	34.0	—	—	441	26	5.9	—	240	4	1.7	—	—
June	981	—	26.0	—	—	458	19	4.1	—	237	6	2.5	—	—
July	868	—	50.0	—	—	430	22	5.1	—	206	13	6.3	—	—
August	841	—	19.0	—	—	470	7	1.5	—	185	3	1.6	—	—
September	893	—	25.0	—	—	489	13	2.7	—	220	0	0	—	—
October	855	—	14.0	—	—	450	6	1.3	—	222	0	0	—	—

* Rates based on total population of last recorded census.

quency of nasal carriers of *Bact. lepisepticum.* Thus, we have available for comparative study mortality, morbidity, and carrier records on several pure line strains of rabbits over a considerable period of time.

RESULTS.

The results of these observations are summarized in Tables I to III, and are described under three main heads,—those pertaining to (1) the entire rabbit population; (2) to various breeds; and (3) to individual animals. We have included only such data as seemed significant; a mass of incidental information collected for the sake of completeness will be referred to briefly.
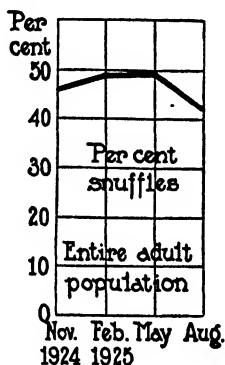
TEXT-FIG. 2. Incidence of clinical snuffles among the adult rabbits at New City farm, N. Y.

Bacteriology.

At the outset, it may be stated that with very few exceptions *Bact. lepisepticum* was obtained from the cases of pneumonia and septicemia examined. It was always recovered from the nasal passages of animals showing clinical evidence of snuffles. Furthermore, a considerable percentage of apparently healthy animals proved to be nasal carriers of this organism.

In every instance the bacterial type obtained was mucoid, with cultural and serological characteristics similar to mucoid strains obtained from rabbits at The Rockefeller Institute and from a rabbit colony at Ray Brook, N. Y. (2). The virulence was uniform and constant; instilled intranasally into young animals, pneumonia was

infrequently induced, snuffles in 40 to 50 per cent of the animals, and "healthy" nasal carriers in 20 to 30 per cent. Detailed studies of some of the strains are described elsewhere (1, *f*).

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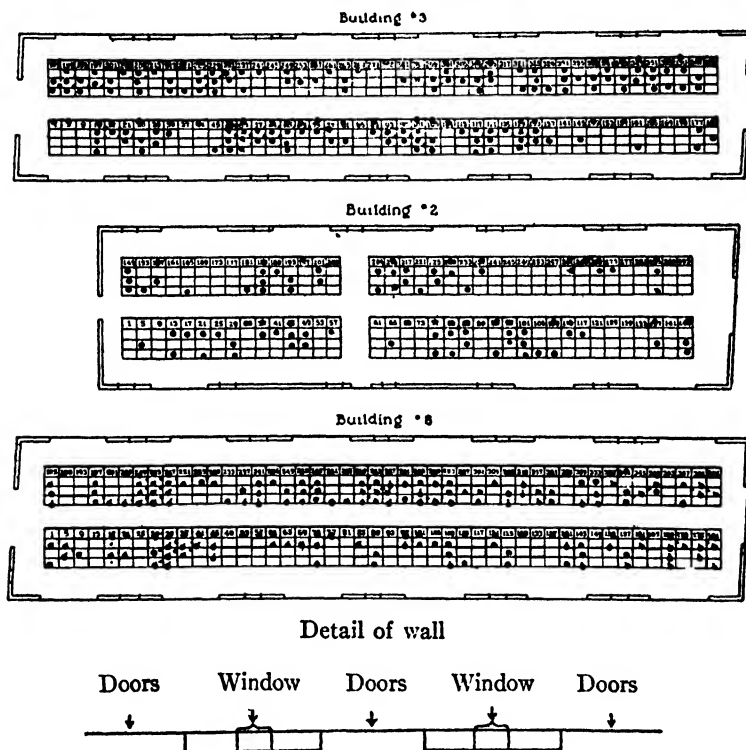
TEXT-FIG. 3.

TEXT-FIGS. 3 to 6. Distribution of clinical snuffles among the adult rabbits at New City farm, N. Y., November, 1924 (Text-fig. 3), February, 1925 (Text-fig. 4), May, 1925 (Text-fig. 5), and August, 1925 (Text-fig. 6).

Findings Referable to the Total Adult Population.

The incidence of clinical snuffles throughout the total adult population was determined in November, 1924, and in February, May, and August, 1925 (Table I). Cases were found to differ in severity and to vary in this respect from month to month. However, all showed the white, purulent, nasal discharge, matted paws, heavy breathing, and sneezing characteristic of *Bact. lepisepticum* infection. Indeed,
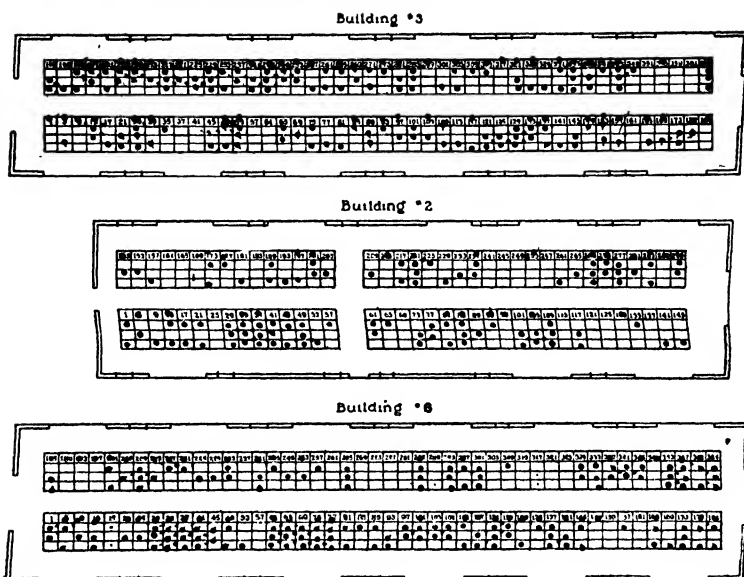
TEXT-FIG. 4. Clinical snuffles, February, 1925.
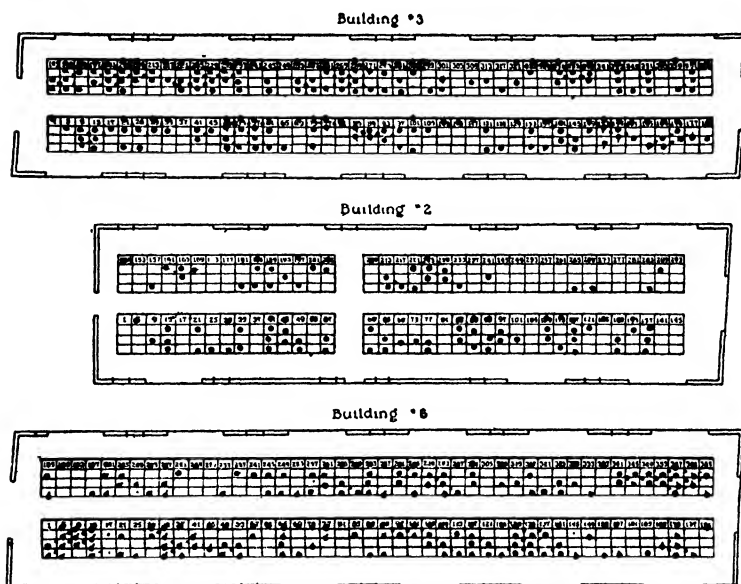
TEXT-FIG. 5. Clinical snuffles, May, 1925.

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whenever cultures were taken from the nasal passages, *Bact. lepisepticum* of the mucoid type (1, *f*) was obtained in great numbers.

The results of these examinations are plotted in Text-fig. 2. The rate per hundred of clinical snuffles rose from 46 in November to 49 in February and May, and dropped to 42 in August. Thus a definite spring rise was noticeable, similar to that observed among the rabbits at The Rockefeller Institute. Among this latter group the average percentage of snuffles varied between about 20 and 60 per cent and exhibited definite spring and fall rises (1, *b*).
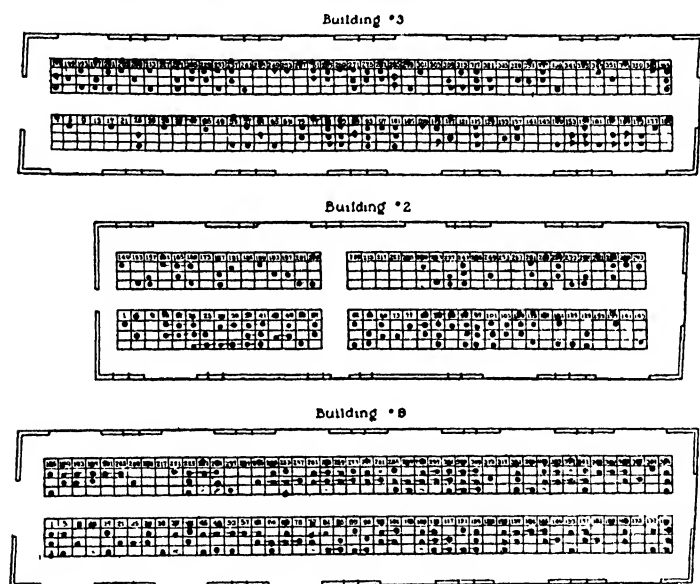
TEXT-FIG. 6. Clinical snuffles, August, 1925.

The distribution of the snuffles cases is charted in Text-figs. 3 to 6. In Text-fig. 3, the spot map for November, 1924, the infection is shown to be present generally throughout the buildings but tending to a focal distribution within each. Thus, in Building 3, definite areas of snuffles are indicated from Cages 13 to 33, 49 to 77, 89 to 109, and 117 to 137. On the opposite tier, there appear to be more cases at either end than in the middle. In Building 2, the infection was less frequent and more scattered. Text-figs. 4 to 6,

which indicate the February, May, and August distribution, show the various foci spreading locally from cage to cage, or disappearing in the same manner.

These maps, together with the mortality records, were useful in demonstrating two facts; first, that *Bact. lepisepitcum* infection in the various buildings was widespread and relatively similar in amount, and second, that the location of doors and windows bore no special relation to the extent or distribution of the disease.

Specific mortality rates from November, 1924, to April, 1925, were obtained by autopsying each rabbit dying within this period and
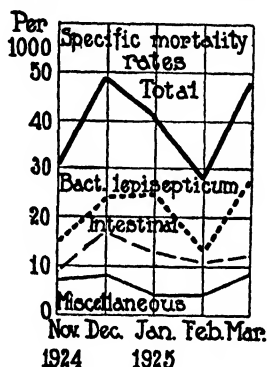
TEXT-FIG. 7. Specific mortality rates among the adult rabbits at New City farm, N. Y.

determining as far as possible the cause of its death (Table I). *Bact. lepisepticum* pneumonia and septicemia proved responsible for more than 50 per cent of the total mortality, intestinal disturbances of undetermined etiology[2] for about 30 per cent, and miscellaneous causes for less than 20 per cent (Text-fig. 7).

Text-fig. 7 shows that the fluctuations in deaths due to *Bact. lepisepticum* coincided with the variations in total mortality. During March, 1925, modifications in the green food diet were instituted and thereafter it became apparent, although no autopsies were performed,

[2] No recognized pathogenic organism was recovered from these cases. Occasionally, pin-worms were present, but not in sufficient numbers to be considered the cause of death. Distention of the ileum, cecum, and colon, with occasional subserous petechiæ and flecks of fibrin were the only pathological findings at autopsy.

that deaths from intestinal disturbances were reduced and that at least 75 per cent of the total mortality was the result of *Bact. lepisepticum* infection. Hence, it will be assumed that the trend of the total mortality curve throughout the 2 year period of observation is a general index of mortality fluctuations due to *Bact. lepisepticum.*

The rate of total mortality per month from November, 1924, to November, 1926, is shown in Text-figs. 8 and 9. During both the 1924–25 period (Text-fig. 8) and the 1925–26 period (Text-fig. 9),
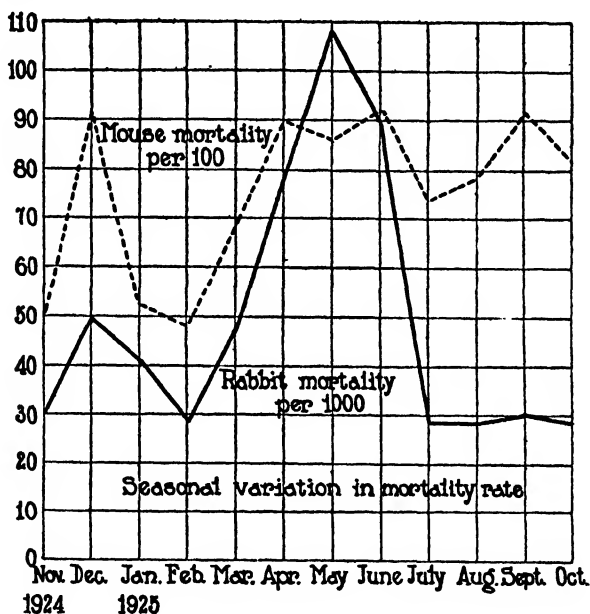
TEXT-FIG. 8. Fluctuations in total mortality of adult rabbits at New City farm, N. Y., 1924–25.

fluctuations occurred in well defined waves, being highest in the spring, low in summer, with a rise again in the fall.

A second curve is included on each chart, representing monthly variations in mortality of mice following the administration of a fixed dose of mouse typhoid bacilli.

The experiments with mice were carried out in such a way as to eliminate as far as possible all disturbing variables (1, *a*). On about the 15th of each month, 50 mice of the inbred Rockefeller Institute strain, 12 to 14 weeks old, weighing

18 to 20 gm. each, bred and raised under the same environmental conditions, given the same diet since birth, and free of any previous exposure to these organisms, were given *per os*, by stomach tube, a fixed dose of about 4,000,000 mouse typhoid bacilli from a standard fluid culture obtained from an agar slant kept at 4°C. (3). Because of the control and elimination of variables in these experiments and because the mortality of a number of groups of similar mice inoculated at the same time differs by a daily average of less than 5 per cent, we consider the wave-like fluctuations in mouse typhoid mortality to be due to variables associated with the season of year.
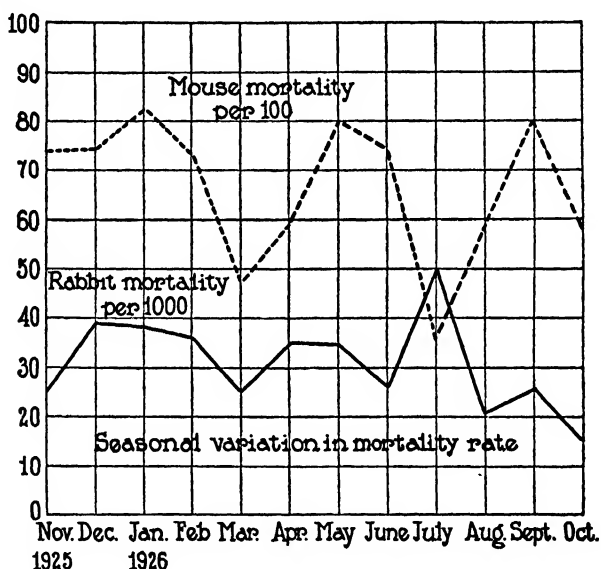
TEXT-FIG. 9. Fluctuations in total mortality of adult rabbits at New City farm, N. Y., 1925–26. From November, 1925, to April, 1926, the mouse curve appears as a fine dotted line. This has been done to indicate a slight change in the technique of the mouse experiments during this period, which changed the absolute values but not the trend.

For 2 years the fluctuations of the mouse typhoid and rabbit mortality curves have shown a remarkable degree of similarity. During 1924–25 (Text-fig. 8) the peaks in December, May, and September, and low points in February and July coincided, while in 1925–26 (Text-fig. 9) the peaks in January, May, and September, and the depressions in November and March were again quite similar. We regard the mouse typhoid curves as quantitative measurements

of the influence of seasonal variables on mortality from mouse typhoid under the conditions of this experiment, and in calling attention to the striking similarity of these curves to the total rabbit mortality in the community at New City,[3] we suggest the possibility that the latter, although relatively uncontrolled, are nevertheless to be interpreted as expressions of variations in host resistance associated with the seasons.

These several observations relative to the total rabbit population may be summarized briefly as follows. The incidence of clinical
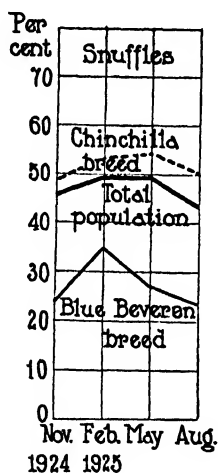
TEXT-FIG. 10.
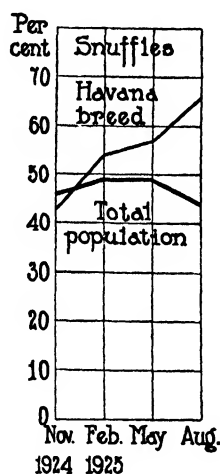
TEXT-FIG. 11.

TEXT-FIG. 10. Comparative incidence of snuffles of Chinchilla and Blue Beveren breeds.

TEXT-FIG. 11. Incidence of snuffles among the Havana breed.

snuffles in the group of about 1000 adult rabbits varied from 46 to 49 to 42 per cent during a period of 10 months, and showed a definite spring rise. The total mortality among these animals, 50 to 75 per cent of which proved to be due to *Bact. lepisepticum* infection, showed wave-like fluctuations over a period of 2 years. High peaks occurred in the spring and fall, and low points were reached in the summer.

[3] The mortality rates of the industrial policy holders of the Metropolitan Life Insurance Company fluctuate in a manner quite similar to the curves in Text-figs. 8 and 9. In them, however, the high spring peak appears in March and April.

Findings Referable to Different Breeds of Rabbits.

The fact that the rabbits composing this population were originally all pedigreed stock of several definite and well established breeds, and that they have continued to be carefully inbred, afforded a remarkable
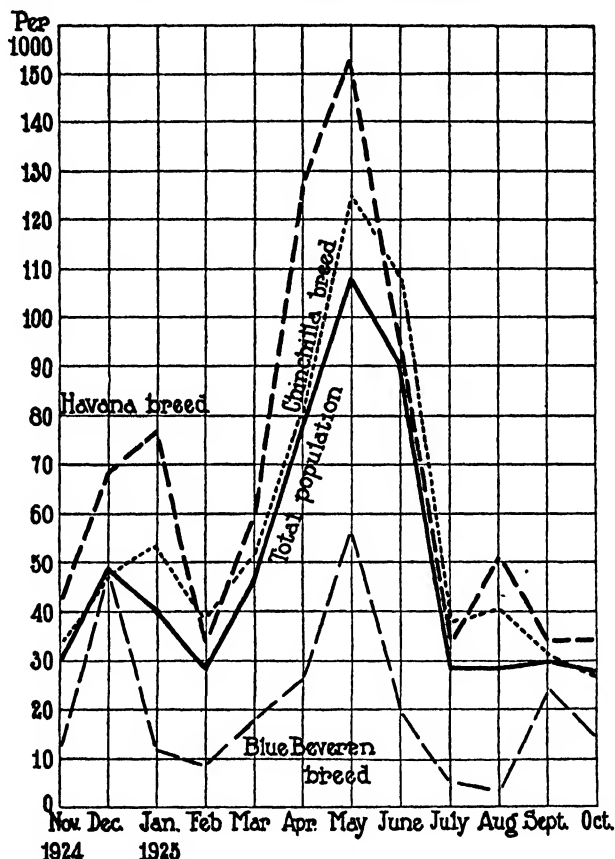
TEXT-FIG. 12. Comparative mortality rates of Chinchilla, Havana, and Blue Beveren breeds, 1924–25.

opportunity to compare the responses of these different animal breeds to a native disease. *Bact. lepisepticum* infection was widespread throughout the four buildings. Probably, therefore, all breeds were continually exposed to a relatively similar dose of organisms. Likewise, other factors inherent in food and environment which might

affect the behavior of individuals or groups toward a harmful agent were relatively uniform. Hence it appeared not unlikely that under these conditions any differences in the response of one or another breed of rabbits to *Bact. lepisepticum* infection could be attributed to factors inherent in that special breed. Text-figs. 10 to 13 and Tables I to III strongly favor this probability in showing that the various breeds do in fact exhibit consistent and significant differences in mortality, occurrence of snuffles, and *Bact. lepisepticum* carrier rates.
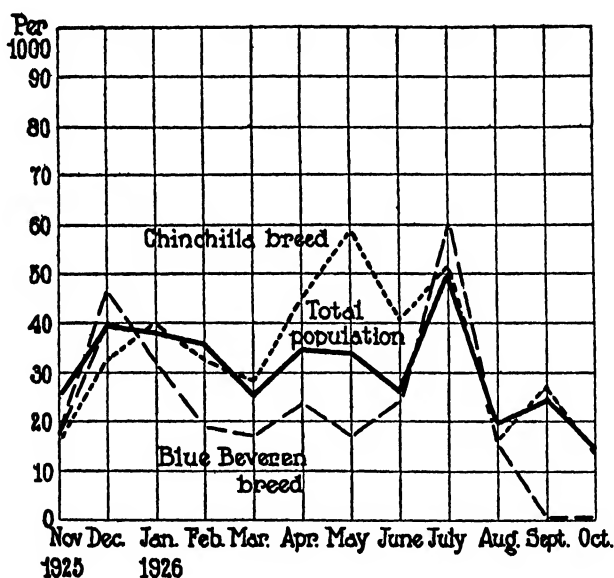
TEXT-FIG. 13. Comparative mortality rates of Chinchilla, Havana, and Blue Beveren breeds, 1925–26.

95 Chinchilla Giants, 158 Belgian hare-Chinchilla crosses, 37 Gaudas, 25 Blue Gaudas, and 14 Blue Flemish animals were studied, but their numbers seemed too small to include in the charts and tables. It is sufficient to state that the snuffles incidence and mortality per cent of the Chinchilla Giants was consistently greater than that of the Chinchillas, that the Belgian hare-Chincihilla crosses were affected to the same degree, and the remainder to a less degree than the Chinchillas.

In Text-fig. 10, the per cent *incidence of clinical snuffles* among about 550 Chinchillas is compared with that of from 82 to 214 Blue Beverens and also with the entire population.   Both breeds showed a fluctuating rate which was highest in the spring.   Throughout the 10 months period, the per cent of infected Chinchillas was nearly twice that of the Blue Beverens.

Text-fig. 11 shows the incidence of snuffles among 117 Havanas. In this case, the rate rose steadily from the general average to a point higher than that for any other breed.

Total mortality per 1000 among these breeds was observed from November, 1924, to November, 1926.   At least 75 per cent was due to *Bact. lepisepticum* infection, hence there are available for comparison 2 years' records of morbidity (Text-figs. 10 and 11) and mortality (Text-figs. 12 and 13) from *Bact. lepisepticum* infection.   The curves in Text-figs. 12 and 13, which describe the total mortality of the various breeds, bring out two facts clearly.   First, that the seasonal fluctuations in mortality of the entire population are reflected in each separate breed—Chinchillas, Havanas, and Blue Beverens show a spring and fall rise and a summer drop in mortality similar to those of the total rabbit population and the experimental mouse typhoid series.   Second, that the mortality·among the Blue Beverens was consistently lower than that of the Chinchillas or Havanas.   Only once during the 1924–25 period (Text-fig. 12), and three times during 1925–26 (Text-fig. 13) did the death rate of the former equal or exceed that of the latter.[4]

Finally, *the carrier rate* among clinically healthy Chinchillas was compared with that among similar Blue Beverens.   The determinations were made as follows.

15 cm. Petri dishes containing 0.5 per cent blood agar were carefully packed and taken by automobile to the New City farm.   Sterile swabs were passed into the nares of selected animals and then streaked over the agar surface of the plates. The cultures were then brought back to The Rockefeller Institute, incubated, and studied in the usual manner (1, *b*, *d*).   Only rabbits free of nasal discharge and matting of the fur on the fore paws were tested.   In all, 142 Chinchillas and 76 Blue Beverens were examined.   Table II shows the results.

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[4] The Havana breed, which suffered the greatest mortality, was so reduced in numbers that their record was discontinued in Text-fig. 13.

42 per cent of the Chinchillas and 26 per cent of the Blue Beverens proved to be carriers of *Bact. lepisepticum* on the first examination. A second and third swabbing of the negative Chinchillas increased their carrier rate to 47 per cent and at the same time indicated the usual 5 to 10 per cent error in the results of a single test (1, *b*, *d*). The relative difference between the two carrier rates—Chinchillas 42 per cent, Blue Beverens 26 per cent—is, however, considerable and entirely consistent with the differences between their morbidity and mortality rates.

In brief, these several observations have shown that the rates of snuffles morbidity and mortality among the special breeds of rabbits at the New City farm display the same seasonal fluctuations as do those of the entire population, and that in regard to incidence of

TABLE II.

Carrier Rate of Bact. lepisepticum among the Chinchilla and Blue Beveren Breeds at the New City Rabbit Farm.

Date	Breed	No. of test	No. of animals tested	Carriers of *Bact. lepisepticum*	
				No.	Per cent
April 18–22, 1925....	Chinchilla	1	142	60	42
"	"	3		67	47
May 7–9, 1925.......	Blue Beveren	1	76	20	26

Bact. lepisepticum carriers, snuffles cases, and mortality, these breeds show definite and consistent differences.

Findings Referable to Different Individual Animals.

For a period of 10 months, records were kept on each individual adult rabbit at the farm. In November, 1924, February, May, and August, 1925, the general condition of each and the presence or absence of snuffles were noted. During this time many animals died, some of which were autopsied, and a number of replacements were made. All these data are of interest but cannot be included in the present communication. We have therefore summarized the records of 828 animals which had been observed from 7 to 10 months and

have grouped them by means of an arbitrary classification into a table (Table III).

The figures were obtained as follows. Only those animals observed from 7 to 10 months were included under "Total number in selected group." In the third column, the entire mortality among these animals during the 10 months period is recorded. Then from a tabulation of the records of the survivors, the cases were classified as follows, in the order of severity of clinical reaction to *Bact. lepisepti-cum:* (1) clinical snuffles, 10 months; (2) clinical snuffles at the outset, disappearing and reappearing; (3) clinically normal at first, later developing well marked and persistent snuffles; (4) clinical snuffles at the outset, later disappearing; (5) clinically normal at the outset, developing snuffles which later disappeared; and finally, (6) clinically normal cases throughout the 10 months period. The occasional otitis media, pneumonia, torticollis, ear canker, diarrhea, etc., cases are omitted from this table because they total less than 1 per cent.

TABLE III.

Reactions of Selected Groups of Rabbits to Bact. lepisepticum.

Breed	Total No. in selected group	Mortality (10 mos.)		Clinical snuffles 10 mos.		Clinical snuffles 10 mos., with remissions		Clinically normal at outset, later developing snuffles		Clinical snuffles at outset, later disappearing		Clinically normal at outset, developing snuffles, later disappearing		Clinically normal, 10 mos.	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Chinchillas.......	578	286	49.5	59	10.2	13	1.8	88	15.2	40	6.9	25	4.3	67	11.6
Havanas.........	144	86	60.0	14	9.7	0	0.0	26	18.5	1	0.7	3	2.1	14	9.8
Blue Beverens....	105	25	23.8	6	5.7	2	1.9	12	11.4	6	5.7	12	11.4	42	40.0

The Havana group showed the most marked reaction to the presence of *Bact. lepisepticum.* 60.0 per cent died, 28.2 per cent had, or developed severe snuffles, and only 9.8 per cent remained clinically normal. Among the Chinchillas as well the effect was severe. 49.5 per cent died, 27.2 per cent showed snuffles during the entire period, or developed it early, and 11.6 per cent appeared clinically normal. The Blue Beverens suffered the least, 40.0 per cent remaining entirely healthy; 19.0 per cent were infected with snuffles at the outset or early during the period, and 23.8 per cent died during the 10 months.

The point deserving special mention, however, is the marked difference in reaction of the individual animals to the presence of *Bact. lepisepticum.* Within each breed there were rabbits which showed no evidence of the disease and others which succumbed; while between these two extremes every gradation was encountered. These differences cannot possibly be due to chance, for it is improbable that the same 67 Chinchillas, the same 14 Havanas, and the same 42 Blue Beverens should have appeared normal on at least four different occasions, over a period of 10 months. Furthermore, the differences in behavior arose under apparently similar environmental conditions and in communities where the bacteria were so widespread that dosage must be considered more or less the same for the entire population. It seems reasonable, therefore, to attribute these variations in animal response, like others which we have noted (1, *g*), to variables inherent in each individual concerned.

DISCUSSION.

The observations on "spontaneous" *Bact. lepisepticum* infection among rabbits have been made under peculiarly advantageous circumstances. Thoroughbred animals, raised in a uniform environment and exposed more or less equally to the risk of infection, comprised the population. Records have been kept of large numbers of individuals over a considerable period of time. The data are therefore relatively free from complicating variables and may be used to advantage in planning and testing the significance of experimental studies.

Investigations dealing with seasonal influences and differences in racial, familial, and individual constitutions show an increasing tendency toward control of complicating variables. Under such circumstances, the more recent findings and interpretations of different observers have been remarkably consistent.

For example, Smiley, in a statistical analysis of the effect of seasonal factors on the incidence of acute respiratory infection, concludes that these infections vary inversely with mean outside temperature and hours of sunlight (4). Chapin, analyzing 102 cases of pneumococcus infection at the Pennsylvania Hospital, notes a relatively high incidence of Type I infection during the winter and early spring months (5). "Common colds" among a group of 6700 clerical employees of

the Metropolitan Life Insurance Company were found to have "two periods of maximum incidence; the first, following the advent of cool weather in the late summer and early fall; the second, occurring during the following January or February when the coldest weather of winter prevails" (6). Schütz in an extensive study of the epidemiology of measles reports that in the large cities of Schleswig-Holstein, the greatest number of cases occur during the spring and fall (7). Pope finds the greatest incidence of scarlet fever to be in January and lowest in July and August but does not think there is evidence of "trend or definite seasonal variation" (8). Rogers attributes the seasonal fluctuations of smallpox in India to the degree of absolute humidity (9). Brown, Pearce, and Van Allen analyzed the first twenty generations of a transplanted rabbit tumor and found that variations in its malignancy tended to follow a seasonal periodicity (10). This they interpreted as due to seasonal fluctuations in animal resistance. Furthermore, by compiling the autopsy data on a large number of so called "normal" rabbits, they found that body and organ weights undergo a definite cyclic change associated with the season (11).

Racial differences in infant mortality in the United States have been studied recently by DePorte. He concludes that the differences in rates of mortality of infants under 1 month of age "very probably have a biological basis and, in this sense, may be termed racial" (12). Meyer and Burghard analyzed the occurrence of scarlet fever and found that family and individual anlages are exhibited definitely by similarity of infection and that the constitution of the individual determines his susceptibility to infection and the course and severity of his disease (13). Lewis (14) and Stevens (15) have noted the same apparent differences in susceptibility to scarlet fever. By means of a skin test with a Shiga dysentery toxin, Brokman and Przesmycki examined 800 individuals for sensitivity. They state that the mechanism of physiological immunity in dysentery is the same as that of diphtheria. It consists in the hereditary capacity to provide normal antitoxin. It is probably a general biological principle which regulates the mechanism of physiological individual immunity against toxogenic bacteria (16). Finally, in a genetic and biometric study of the "Constitutional element in the etiology of pneumonia," Pearl (17) describes a pedigree which has in the father's kinship—"a group of people with a definite tendency towards constitutional inferiority of the respiratory system, which manifests itself chiefly in a tendency to break down from pulmonary tuberculosis in early adult life. This particular constitutional inferiority is absent in the mother's kinship, but in that group of people there is definitely manifest a constitutional tendency to generally non-fatal respiratory infections, bronchitis and bronchopneumonia, in infancy and childhood. When these two constitutional traits were combined, by the mating of the father and mother of the 13 children in the sibship under discussion, there was produced a group of children with extremely low resistance to any sort of respiratory infection, with a consequent 100 per cent incidence of pneumonia in the years of infancy and childhood."

These references to recent literature will perhaps indicate the present trend of thought on these problems. The data are with one exception, which follows, purely descriptive, and have inherent in them a number of indeterminable variables which for the most part have been dealt with according to statistical methods. A direct experimental proof of seasonal variation in mouse typhoid infection has, however, been offered, under conditions in which every known variable except season has been eliminated or controlled (3, *a*). Also racial differences in susceptibility to this infection have been proved experimentally (3, *b*) as well as wide differences in the susceptibility of individuals (1, *h*). Tests with *Bact. lepisepticum* have also furnished evidence that individual rabbits differ in their susceptibility to this disease (1, *i*). Hence the descriptive data are in no small measure supported by the more precise results of direct experiment.

From the general epidemiological view-point, the observations recorded in this paper are of interest in that they describe a rabbit community in which a bacterial infection was endemic and where the type and virulence of the microorganisms concerned were tested from time to time and found to be uniform and constant. A similar study of a rabbit community in which a severe epidemic occurred is reported in the following paper by D. T. Smith (2). Types of *Bact. lepisepticum* recovered by him at Saranac resembled in every respect those occurring at New City. Okamoto, studying epidemics among laboratory animals, found bacterial virulence to be relatively constant (18). R. Freund, investigating a mixed Pasteurella, pneumococcus, and paratyphoid epidemic among a stock of rabbits and guinea pigs at the Robert Koch Institute in Berlin, found no differences in the virulence of various epidemic strains, and concluded that the cause of the outbreaks was due to an increase in population susceptibility following sudden and severe changes in temperature (19). Finally, Theobald Smith and his collaborators have studied a paratyphoid epidemic among a stock of guinea pigs and found no definite fluctuations or differences in the virulence of the specific bacteria. They, too, conclude that changes in population susceptibility must be regarded as the most probable cause of the outbreak (20). Several populations, therefore, have been observed by different investigators to be infected with intestinal and respiratory diseases which have

undergone epidemic and endemic fluctuations in prevalence and severity without any demonstrable change in the virulence or type of the bacteria concerned.

SUMMARY.

The spread of *Bact. lepisepticum* infection throughout a population of thoroughbred rabbits at a farm in New City, N. Y., was studied over a period of 2 years.   The following observations are noteworthy.

1. 50 to 75 per cent of the total mortality was due to *Bact. lepisepticum* infection.

2. About 50 per cent of the stock was affected locally with *Bact. lepisepticum* "snuffles."

3. "Snuffles" and mortality rates showed spring and fall rises and low points during the summer.

4. Carrier, morbidity, and mortality rates of various special breeds differed consistently and to a marked degree.   All reflected the same seasonal fluctuations as the total population.

5. Individuals within each breed differed in their response to the presence of *Bact. lepisepticum;* some died of penumonia and septicemia; others developed the local nasal infection, "snuffles;" others became "healthy" carriers; and a few remained uninfected.

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EPIDEMIC ENCEPHALITIS AND SIMPLE HERPES.

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The pandemic of encephalitis which encircled the world between 1916 and 1920 brought a new problem into epidemiology and pathology. Taking its beginning inconspicuously, probably in Austria in 1916–17, the disease first named lethargic encephalitis (von Economo), was observed next in France and England in 1918 and later (1919–20) in the United States and other more remote countries (1). The indications are that the disease appeared in mass first in Eastern Europe, passing thence to Western Europe, America, and the Far East. While the disease has died down in, although it has not disappeared from European countries and America, it has prevailed in force in Eastern countries within the past 2 years. Japan was visited by an epidemic of encephalitis in 1924, in which 6000 or more cases were reported (2). This large recent outbreak was characterized by a high mortality (reaching 70 per cent), while the mortality of the earlier European and American malady was about 25 per cent. In the absence of an established specific cause for the epidemic outbreaks, the question remains whether all were of the same nature, or whether several distinct diseases have, for unknown reasons, been attended by a high proportion of brain complications or sequels.

The wide prevalence of an epidemic disease presenting novel clinical features gave rise at once to speculations such as, (*a*) the previous occurrence, and (*b*) the nature and causation or etiology. While encephalitis, or brain inflammation, is known to attend certain definite diseases, among which are epidemic influenza, ulcerative endocarditis, and epidemic cerebrospinal meningitis, yet as such attendants, the number of cases arising is relatively not large. Along with the other peculiarities of the epidemics of encephalitis of this period are

to be mentioned, therefore, the wide occurrence, the large number of cases, and the absence of close correlation with epidemics of influenza.

The earlier literature does not contain records of parallel outbreaks of encephalitis. Perhaps the most frequent precursor or concomitant of small prevalences of encephalitis, so far as the records show, is epidemic influenza. A pandemic of epidemic influenza of extreme severity swept over the world between 1917 and 1920. The question arises immediately, therefore, whether the two epidemic maladies— influenza and encephalitis—have, in this instance, been connected in a causal way.

In endeavoring to supply a definite answer to this question, we are in the difficult position of having no certain knowledge of the nature of the microbic incitant of influenza. Were we in possession of this much sought knowledge, we should probably be able to state immediately whether influenza and encephalitis are induced by the same microbe, acting chiefly on different parts or organs of the body. In the absence of this knowledge, the answer to be given to the question must be indirect and hence perhaps not final.

This significant question has been examined in detail elsewhere (1), and the conclusion reached is: "The pandemic of lethargic encephalitis of the second decade of the twentieth century is quite unprecedented in recorded medical history, and is probably not merely an unusual nervous manifestation of epidemic influenza, but is rather an independent affection, etiologically considered, to be compared in its specific nature with other defined pathologic entities, such as typhoid fever, tuberculosis, poliomyelitis and epidemic meningitis."

Encephalitis.

The kind of inflammation of the brain to which the name encephalitis is applied is a not uncommon pathological condition in man and other mammals. In man, the commonest varieties are met with in African sleeping sickness, a disease incited by trypanosomes; and in syphilis, a disease incited by the spiral organism *Treponema pallidum*. Encephalitis is also an attendant of poliomyelitis in man, although in this disease the brain is less often affected than the spinal cord, and the high degree of affection of the one usually obscures the slighter involvement of the other. Aside from the particular and specific forms

of encephalitis in man there occur the cases already mentioned as complicating epidemic influenza, endocarditis, and epidemic meningitis, as well as certain other grouped cases of still uncertain nature, as represented by the so called Australian X disease (3).

Other mammals, namely horses, dogs, rabbits, and doubtless still other species, are subject to particular kinds of encephalitis. The prevailing form in horses, often reaching epidemic proportions, is the so called Borna's disease; while many domestic stocks of rabbits show encephalitis, partly incited by the protozoan parasite *Encephalitozoon cuniculi*, and partly perhaps by other microbes (4). Encephalitis is not infrequently detected in dogs succumbing to distemper and to rabies.

This pathological condition, widely present in mammals, shows a striking uniformity of microscopic characters in all the species affected. Two classes of lesions are met with: degenerative and infiltrative. The first consists of various destructive changes in nerve cells and supporting (glia) tissues. The second consists of cellular invasions most conspicuous about small blood vessels and in the adjoining nervous tissues. The nerve cells consist chiefly of the mononuclear elements called lymphocytes. They tend to be heaped about the blood vessels and to form small aggregations in the tissues themselves. This peculiar distribution of the invading lymphocytic cells gives a striking character to the microscopic appearances, especially revealed in African sleeping sickness and lethargic encephalitis in man, and in Borna's disease and the so called spontaneous encephalitis in rabbits (5).

Experimental Encephalitis.

The method followed in investigating epidemic encephalitis experimentally was to inoculate rabbits and monkeys with brain tissue and cerebrospinal fluid taken from cases of the disease in man. The inoculations were usually made by intracerebral injections. These animals withstand such injections very well and show no symptoms referable to the inoculation, unless infection occurs. The infection which may occur arises (a) from specific organisms, as in experimental poliomyelitis; or (b) from accidental secondary, usually pyogenic organisms. The two resulting conditions are readily distinguishable.

There is general agreement among investigators that the monkey is not subject to inoculation with materials taken from the central nervous system of cases of epidemic encephalitis. The rarely successful inoculation reported may be interpreted as produced by an extraneous cause (1).

An active controversy, not yet wholly composed, has centered about the result of the inoculation of rabbits. While certain investigators succeeded in producing in rabbits encephalitis which they identified with epidemic encephalitis in man, other equally experienced ones failed wholly to obtain unequivocal results. A part of the disparity has been explained by the discovery of the frequence of spontaneous encephalitis in the domestic rabbit; in this way the early, supposedly successful experiments of Loewe, Hirshfeld, and Strauss (6) may be accounted for. Another part of the disparity may be explained by the discovery that the clear contents of herpes vesicles in man contain an active substance, believed to be a living, submicroscopic organism, so called virus, which is inoculable in rabbits, in which animals an encephalitis, usually fatal, is often induced (7).

Experimental Herpes.

Simple or febrile herpes is a common and innocent affection in human beings. The eruption consisting of vesicles to which this name is given usually appears on the lips. Certain persons are so subject to the eruption that very slight maladies, simple colds, etc., frequently produce an outbreak. On the other hand, the rabbit as far as is known is not naturally subject to a corresponding affection. And yet, when the clear, non-bacteria-containing, vesicular contents are inoculated into rabbits, profound effects are produced. If the contents are inoculated into the scarified cornea, severe kerato-conjunctivitis arises; if into the scarified skin, vesicular dermatitis follows; if into the brain, fatal encephalitis results. In some instances the corneal inoculations are followed by the nervous symptoms and fatal effects of an encephalitis; the skin inoculation by symptoms and effects of a myelitis and subsequent encephalitis; and injection of the virus into the testis also by a fatal encephalitis.

When the symptoms and effects on the brain of the experimental

herpes rabbits are compared with the few instances of actual successful inoculation of rabbits with nervous materials taken from human cases of epidemic encephalitis, it has been found that the two sets of conditions are indistinguishable. And in keeping with this observation it is also found that the so called virus of encephalitis, as contained in the brain of rabbits, is inoculable upon the cornea and the skin of these animals, in which it produces vesicular inflammations corresponding accurately to those set up by the herpes virus. Hence two questions at once suggest themselves: (a) Are the herpes virus and the supposedly encephalitis virus identical; and (b) if identical, is epidemic encephalitis merely a manifestation of the location in and action of the common herpes virus upon the brain of man, made possible by particular and thus far undiscovered conditions which have prevailed fitfully in different parts of the world from 1916 up to the present time (8)?

Two points of view are being entertained regarding these questions: one represented by Levaditi and to a less extent by Doerr (9), according to which epidemic encephalitis is a manifestation of the action of special strains of herpes virus acting on the prepared, susceptible nervous system of man; and the other represented by Flexner and Amoss (10), who hold that the true virus of epidemic encephalitis remains undiscovered, and that the small number of successful inoculations of human encephalitic materials reported can be explained on the supposition that there was present in these an accidental admixture of herpes virus.

That this kind of admixture actually may occur is shown by an instance in which herpetic encephalitis was induced in the rabbit by the injection of the cerebrospinal fluid taken from a case of neurosyphilis showing at no time symptoms of epidemic encephalitis. This strain of herpes virus produces all the inoculation effects and is biologically indistinguishable from the ordinary herpes and the so called encephalitis virus (11).

Recent Experimental Findings.

The discussion regarding the etiology of epidemic encephalitis and the relationship existing between herpes and the so called encephalitis viruses, has recently centered about certain anomalous ex-

periments made on guinea pigs as reported by Rose and Walthard (12) and by Dmitrieff (13). In order to follow the views of these authors, it is necessary to recall the fact, established by many investigations, that while the rabbit is highly susceptible to inoculation with the herpes group of viruses, the guinea pig, the rat, mouse, and other rodents, are far more resistant. Other species—dog, cat, monkey—are practically wholly insusceptible.

Although the rabbit is so sensitive to the inoculation, yet in order to implant a virus on this animal from materials taken from cases of epidemic encephalitis, the rule is to inject several animals, so that the differences in individual sensitivity naturally existing may be compensated. When the inoculation has succeeded, which has rarely been the case, usually one animal in the series develops symptoms. As has already been stated, the great majority of investigators have not succeeded in obtaining any transmission whatever to the rabbit.

The reported experiments with guinea pigs are supposed to explain this anomaly. In effect they are as follows: When a strain of the herpes-encephalitis virus is injected intracerebrally into guinea pigs, after an incubation period of several days, an encephalitis arises. The symptoms and effects of the encephalitis produced resemble those of the rabbit, although they tend to be less severe. Rabbits when inoculated intracerebrally with an active strain of virus not only develop encephalitis, but regularly succumb to the disease. The guinea pig, on the contrary, is said to tend to recover from the encephalitis and animal to animal transmission to be impracticable. Indeed, according to Rose and Walthard and to Dmitrieff, even when encephalitis is produced in the guinea pig, the introduced virus is rapidly destroyed. It is this rapid destruction within the susceptible brain tissue which led the authors to venture the opinion that similarity exists between the effect of the herpes-encephalitis group of viruses on the brain of man and of the guinea pig, and to see in this supposed similarity support for the belief that epidemic encephalitis in man results from infection with a virus of the herpes-encephalitis kind.

The observations reported by Rose and Walthard and by Dmitrieff should as such be accepted as a fact, although the number of experiments performed is not large. In Dmitrieff's case the number is indeed very small. The question which the experiments do not an-

swer and which is yet decisive is, whether the results obtained apply to one or two strains only, or to all strains of the herpes-encephalitis virus. Flexner and Amoss (14) have shown that, as measured by rabbit inoculations, what may be called strong and weak strains of the virus exist. It is noteworthy that the Levaditi and Doerr strains of the virus, supposedly of encephalitic origin, belong to the weak class (15). Rose and Walthard used a Doerr strain in their tests. Hence it becomes important to ascertain what happens when a strong or highly virulent strain of the virus is injected intracerebrally into guinea pigs.

We possess in the H. F. strain such a strong herpes virus (14). With this virus we have infected guinea pigs by intracerebral and intra-corneal inoculations through a series of passages which, at the termi-nation of the 10th cerebral transfer, showed no diminution of activity and no lengthening of the incubation period. If anything extra-ordinary occurred in the course of these passages, it was an adaptation of the virus to the guinea pig, which regularized its activity by making the inoculation effects more uniform and certain as well as of shorter duration. The cerebral injections were performed in pairs, and while at first the two pigs might develop symptoms a few days apart, in the later passages they often developed them simultaneously; unless sacrificed for experimental purposes, these pigs succumbed.

The symptoms were typical of experimental herpes virus encephali-tis and included high temperature (up to 106.5°F.), paralysis, and salivation. The fact should be emphasized especially, since the observation is a new one, that the inoculation of the cornea not only induced kerato-conjunctivitis, but also in a number of instances a fatal encephalitis. The following is a protocol of an experiment of this kind.

Protocol.

Nov. 4, 1926. Right eye of guinea pig cocainized and scarified with cataract knife dipped in a 10 per cent suspension of fresh guinea pig brain of animal which reacted to intracerebral inoculation of H. F. virus. Nov. 6. Beginning kerato-conjunctivitis; temperature 104.6°F. Nov. 7. Opacity and small vesicles on cornea; inflammation increased; temperature 105°F. Nov. 8. Eyelids glued together; temperature 106.2°F. Nov. 10. Temperature 105.2°F.; turns to right. Nov. 11. Tremor, ataxia, falls easily. Nov. 12. Temperature 106°F.;

tremor and ataxia increased. Nov. 13. Symptoms advanced; salivation; temperature 106.4°F. Nov. 16. Death.

That it is possible to excite a severe kerato-conjunctivitis in the guinea pig by eye to eye inoculation is shown by the pig inoculated from the eye of the above named animal on Nov. 8. This animal developed typical progressive inflammation of the cornea and conjunctiva, and on Nov. 13, 24 hours after the temperature had reached 106.2°F., began turning to the right side (side of inoculation). Tremor, ataxia, convulsions, and salivation ensued, and death occurred on Nov. 18, or 10 days after the inoculation.

Penetration of the virus from the eye to the brain of guinea pigs did not always occur. The behavior of the corneally infected pigs was often typical of the rule, namely the inflammatory reaction would be less severe than is observed in rabbits, and with its abatement the eye would return to normal. The cornea tended not to become opaque and the eyeball shrunken.

The employment for the inoculation of guinea pigs of a strain of herpes virus shown by rabbit tests to be strong, permits of the drawing of entirely different conclusions from those reached by Rose and Walthard and by Dmitrieff, both of whom used weaker strains. What has been described by Rose and Walthard and by Dmitrieff are only special instances of the action of such weak strains of the virus in guinea pigs. Moreover, a comparison of the experiments of Rose and Walthard with those of Dmitrieff suggests that the strain of virus used by the latter was definitely weaker than that employed by the former, and more quickly suppressed by the guinea pigs. Indeed, in view of the history of many cases of epidemic encephalitis in man in which the disease pursues a progressively degenerative course, it would seem almost inevitable that the inciting microbic agent, far from being quickly destroyed, actually possesses the power of continuous, slow multiplication, because of which the pathological processes fail to be arrested.

Contradictions in Etiologic Findings.

Epidemic diseases show, as a rule, identical microbic incitants, irrespective of the time and place of their prevalence. Once the microbe has been discovered and shown beyond doubt to be the incitant, then the epidemic disease, whether appearing in Europe, America, Asia, or Africa, has always been accompanied by particular

microbes, the biological properties of which are indistinguishable in all essential respects.

If this test of correspondence is applied to the agents obtained during outbreaks of epidemic encephalitis, it fails to hold. Attention has already been drawn to the fact that in only three certain instances (Levaditi, Doerr, Schnabel) (1) has an herpes-encephalitis strain of virus been obtained from cases of epidemic encephalitis, while many more investigators have failed altogether in their search for this virus. On the other hand, still other investigators have reported the isolation of kinds of virus which differ from the herpes-encephalitis variety, and these exceptional specimens fail to agree with one another. The outstanding exceptional findings are those of Kling of Sweden and of Takaki of Japan.

In both instances, disease was induced in rabbits through injecting materials derived from cases of human encephalitis. The experimental disease described by Kling differs wholly from that described by all other successful investigators, in that it is not an acute, but a chronic pathological process. A critical examination of Kling's results has been made elsewhere (1). It remains to examine the results obtained by Takaki (2).

In the summer of 1924, about 6000 cases of epidemic encephalitis were reported from different parts of Japan. Transmission experiments were undertaken, and both failure and success were reported. Perhaps the most notable instance of success is that of Takaki, who reports 6 transmissions of a virus disease to rabbits with autopsy material from 6 fatal cases. The virus cannot be cultivated artificially; it is inoculable by way of the cornea, brain, and other organs. The eye effects, however, differ from those of the herpes virus effects; the general symptoms, which include paralysis, but not excitement, also differ from those of the herpes virus effects. The symptoms, therefore, as exhibited by rabbits do not correspond to the symptoms produced by inoculation of the herpes-encephalitis group of viruses. Moreover, comparison of the Japanese virus with the herpes-encephalitis virus through immunity tests and reactions, shows it to be dissimilar (2).

In view of this discordant finding, the question arises whether the Japanese and the European epidemic diseases are pathologically the

same. Fortunately this question can be answered, and apparently in the affirmative. The clinical and pathological descriptions which have been published show close similarity. Through the kindness of Professor Kimura, of the Imperial University in Sendai, I have been enabled to examine specimens taken from the brain of fatal cases. These specimens show pathological changes closely resembling those found in the brain of Europeans and Americans who have succumbed to epidemic encephalitis. The changes or lesions are of two sorts: monocellular (lymphoid) infiltrations of the blood vascular sheaths and brain tissue, and degeneration of ganglion and glia cells. The distribution of the lesions is also typical. Especial attention may be drawn to the lesions of the substantia nigra which are prominently present in the Japanese, as well as in the European cases of the disease.

There is no doubt that the Swedish cases of epidemic encephalitis are identical with the other European and the American cases. From what has just been stated, there are strong reasons for believing that the Japanese epidemic disease is of the nature of the European and American disease. The essential differences relate to the microbic incitants described by Kling and by Takaki. As tested by these discrepancies, the epidemics would have to be regarded as distinct. The fundamental question raised by the discrepancies is, therefore, whether the experimental findings are not open to the suspicion of not revealing the real incitant of the epidemic disease. What must also be taken into account is the possibility of other circumstances coming into play, such as unrecognized, preexisting disease of the rabbit, or of the operation of contaminating organisms which produce effects in the inoculated animals and yet play no part in the human epidemic disease.

Native Animal Viruses.

The employment of the rabbit for the experimental investigation of epidemic encephalitis has led to a state of confusion not yet terminated. That the earlier observations of Loewe, Hirshfeld, and Strauss (6), Kling (16), and some others were vitiated by the presence of an unrecognized, preinoculation form of encephalitis in these animals, is now either proven or probable. Nishibe (17) has recently

shown that the domestic rabbit of Japan likewise suffers from preinoculation encephalitis, and hence is unsuited for the study of the perivascular and other infiltrative lesions of the brain. It may be queried whether Nishibe is more fortunate in citing as evidences of experimental infection, the more subtle lesions of nerve cells of the rabbit's brain, which he regards as indicative of this condition. In emphasizing these lesions and in discarding the infiltrations as indications of a state of encephalitis, he is introducing not only new considerations in respect to the experimental disease, but is also setting up a class of lesions as criteria which has not yet been described for the human affection.

The difficulties encountered by the use of the rabbit and some other animals for the experimental investigation of epidemic encephalitis do not end here. There can be small doubt that in certain instances, what appear to be transferable virulent materials, differing from ordinary bacteria, have been obtained in the course of the inoculation of rabbits and of dogs with human nervous tissue. These active materials, called by their discoverers "viruses," and believed by them to be concerned with the production of encephalitis, are not all, as has already been pointed out, of one kind. The virus of Koritschoner (18) certainly, and that of Kobayashi (19) probably, are merely strains of rabic virus, while the virus called Eck (20), by reason of the fact that it was recovered from dogs in which an Eck fistula had been produced, has not yet been identified with a known variety. One can only speculate on the source of these virulent agents. Where dogs are employed, it is always possible that a latent rabic virus may come to be isolated in the course of the transfer of nervous tissue. Where autopsy material from human cases is used for inoculation, something may depend upon the way it was removed from the body and how it was treated in order to guard against even gross contamination.

However this may be, it would seem that these explanations do not entirely cover the observations of Takaki (2). The virus obtained by him several times from 6 fatal cases of the Japanese encephalitis, differs apparently from all those hitherto studied. It does not belong to the herpes-encephalitis group, and apparently not to the rabic viruses. Since the Takaki virus was obtained from the cases of epi-

demic encephalitis studied histologically by Kimura,[1] there is no reason to doubt that it came, directly or indirectly, from definite encephalitic material.   What is noteworthy, however, is that there is fundamental lack of agreement among the Japanese investigators of the epidemic disease of Japan, just as there is lack of uniformity between their observations and those of European investigators of epidemic encephalitis.

We must leave the clearing up of these discrepancies to further investigations of the native viruses of domestic animals.   Thus far almost no heed has been paid to the existence in animals in a state of nature, of virulent agents, possibly true viruses, which remain in a condition of latency until through some simple device they are made to assert themselves.   The best example of this class of substances now known is that described by Rivers and Tillett (21) for the rabbit. An analogous example is the one described for the guinea pig by Jackson (22) and by Cole and Kuttner (23).   It would seem as if we are at the very beginning of knowledge of this class of potential pathogenic agents; and it would also seem that we shall have to take into account the possibility of a greater number and wider distribution in animals ordinarily called "normal," of these agents in connection with the experimental study by animal inoculation of human diseases, the etiology of which is still undiscovered.

CONCLUSIONS.

The purpose of this paper is to explain the state of our knowledge of the etiology of epidemic encephalitis, and especially to draw a line of demarcation between the established virus of simple herpes and the hypothetical virus of epidemic encephalitis.   It had already been shown that the experimental observations on rabbits do no suffice to prove the identity of the herpes with the encephalitis virus.   The discussion of the subject in this paper shows that identity cannot be postulated on the basis of the performed guinea pig experiments.   Attention has been drawn to the significant fact that there is lack of harmony in the positive results of those investigators who believe that the

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[1] We are indebted to Professor Kimura for specimens of human brain tissues which show definitely lesions that are indistinguishable from those occurring in the brain of European and American cases of epidemic encephalitis.

incitants of epidemic encephalitis have been discovered. An attempt has been made to attribute some of the discrepancies reported by these investigators either to accidental and contaminating microbic agents, or to the uncovering of virulent agents preexisting in a latent state in the animals employed for inoculation, the existence of which was not previously known or suspected. Since past experience leads us to believe in a single incitant for widespread epidemic diseases, it is probable that, when certainly discovered, the microbe of epidemic encephalitis will prove to be simple and not multiple. The direct corollary to this point of view is that up to the present, the etiology of epidemic encephalitis has not been determined.

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SUMMARY OF OBSERVATIONS OF THE COMMISSION TO STUDY FOOT-AND-MOUTH DISEASE.*

BY PETER K. OLITSKY.

(From the Laboratories of the Commission to Study Foot-and-Mouth Disease of the United States Department of Agriculture, at the Institut d'Hygiène et Bactériologie, Strasbourg, France.)

The Commission to Study Foot-and-Mouth Disease was formed by the United States department of agriculture, and comprised Dr. Harry W. Schoening, of the bureau of animal industry; Dr. Jacob Traum, of the University of California, and Dr. Peter K. Olitsky, of The Rockefeller Institute for Medical Research. Dr. Louis Boëz, of the Institut d'Hygiène, Strasbourg, France, where most of the work was done, acted as collaborator. We owe many thanks to the Director of the Institute, Professor A. Borrel, for his cooperation. The carrier problem was studied at Alfort, France, with the generous and helpful assistance of Professor Vallée.

The work of the Commission will be presented in detail in a forthcoming publication by the bureau of animal industry. A preliminary article has already appeared in the *Journal of the American Medical Veterinary Association*, 1926 (November), lxx (N. S. xxiii), 147, in which were reported comparative studies on vesicular stomatitis and foot-and-mouth disease. A summary of the more important observations of the Commission will now be given.

Transfer of the Virus to Guinea Pigs.

Guinea pigs were successful and regularly inoculated with suspensions of the virus by puncturing, scarifying, and "tunneling" the epidermis of the hairless posterior pads. The disease in these rodents is almost a counterpart of that in bovines. Primary vesicles appear at the point of inoculation within twenty-four to forty-eight hours after injection, which are followed in a day or two by secondary

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vesicles in the uninoculated mucosa of the mouth, or in the tongue, or in the anterior pads. Several bovine strains were transferred to guinea pigs, and of these one was propagated in the latter animals for 261 passages, during about one year. Thus the guinea pig offers an aid to diagnosis and laboratory studies on the virus, and is suitable for its propagation. Furthermore, there is no difficulty in inducing foot-and-mouth disease in cattle and hogs with guinea pig virus (Strasbourg, Riems, and Vallée A strains) by either local or intramuscular inoculations of cattle and by intravenous injection of hogs. The disease so induced is similar to the natural affection. However, while in nature the disease is most contagious, among guinea pigs, there is practically no communicability.

Plurality of Types of the Virus.

Of greatest importance to the efficacy of control measures, to epizootic conditions, and to investigations on immunity is the presence of more than one type of the virus. We have found that there are at least two types of foot-and-mouth disease virus corresponding to Vallée's Types O and A. These do not cross-immunize, as noted by tests on cattle, hogs, and guinea pigs, nor do they induce a clinical picture by which one can be distinguished from the other.

The Presence of the Virus in and outside of the Animal.

The virus is contained in the blood during the initial febrile stage of the disease. It is also present in the fluid, and in the coverings of the vesicles. In the vesicles of cows and hogs the virus was active for guinea pigs for not longer than seven days after the first appearance of symptoms. In most instances, however, such materials were inactive after the fourth day. We found the virus in saliva. The urine from only two cows was tested with negative results. We did not search for the virus in milk or feces. Bielang was the only investigator to study the latter material, and reported the absence of the active agent in the feces of three cattle and five hogs, examined from twenty-four to one hundred and twenty hours after artificial infection. These experiments are too few in number to permit generalizations, but the repeated findings of others, however, indicate that the virus

may be present at one time or another in practically all the secretions and excretions. Whether this is due to admixture of these materials with vesicular contents or coverings is immaterial; the practical bearing on epizootics and on control measures is important, and that is, that the secretions and excretions should be considered as harboring the virus, at least in the early stages of the disease. Furthermore, we have found that cattle contain virus in the oral secretions, even before the first symptoms of the disease are made manifest.

With respect to the viability of the virus outside the body, the conditions under which it is kept determine in a large measure its resistance to destruction. In the laboratory, at 37°C., the active agent dies within twenty-four to forty-eight hours. This has given rise to an impression that the virus is quite fragile. But it is merely an extraordinary phenomenon, still inexplicable, and does not indicate the true character—the remarkable resistance—of the virus. For at room temperature we have kept the virus alive for longer than 69, but shorter than 100 days. In the cold, in 50 per cent glycerol, it is preserved indefinitely. Under field conditions, we have noted that in hay or in garden soil the virus remains living for at least 25 to 30 days. That the active agent is not rapidly destroyed after leaving the animal is substantiated by the American instance in which the incitant persisted in the field for one year. Hence, we conclude that it is of utmost significance from the standpoint of spread of the disease and its control, to regard the virus as resistant, and not as fragile, as some others believe.

Carriers

The presence of carriers was studied experimentally. In one series of tests, twenty selected, recovered Swiss cattle, presumed to be possible carriers of foot-and-mouth disease virus by Swiss federal veterinary officials, were placed in contact with twenty-eight normal cattle and four hogs of French origin, and seven normal cattle from Switzerland, believed by the local authorities to be more susceptible. About one month before this experiment was terminated, the hoofs of the suspected carriers were pared and scraped so as to make the test more rigid. After three to four months' contact, no disease occurred in the normal animals, although at the end of this period

they were proved to be susceptible to both "O" and "A" types of the virus.

This experiment, performed under the most elaborate conditions and carefully controlled, failed. But a negative result under the circumstances is inconclusive. The questions arise as to whether sufficient animals were employed, or whether the time of contact was long enough for an unequivocal test. In the next series of experiments, somewhat different results were obtained.

The hoofs of twenty-two cattle and one hog were examined at postmortem twenty days to six months after the onset of the disease. The hoofs were sectioned with a saw, and pockets, double soles, and the tissues generally were scraped. The scrapings were then inoculated into guinea pigs. The material from only one of these animals, slaughtered thirty-four days after inoculation with the foot-and-mouth disease virus, was positive. It appears, therefore, that one of twenty-three animals harbored active virus thirty-four days after infection.

After consideration of both tests, one may conclude that in a very small number of cases, a recovered animal may carry the virus. Actual experimentation reveals that the number is much smaller than that believed by some authorities. On the other hand, it may explain in part the isolated outbreaks due to carriers reported by the Italian, British, and Swiss officials.

At this point we may state that we have failed to implicate the earthworm as a carrier.

Immunity.

This problem was studied in guinea pigs and cattle. The limitation of our time prevented us from studying the duration of protection, but we have found that a solid immunity, but only against the homologous type, either "O" or "A," is present for at least three to four months after infection. Gins reported a case in which resistance to infection was maintained for 387 days. More work is necessary on the problem of duration, since it is now known that there is more than one strain of virus.

The immunity in experimental foot-and-mouth disease is of an extraordinary type. Serum from recovered animals, either from

cattle or guinea pigs, or hyperimmune serum, does not prevent the occurrence of primary lesions; it inhibits the development only of secondary vesicles. This results if the serum is injected in the guinea pig either subcutaneously or locally by a puncture-scarification method. On the other hand, if a proper inoculation of virus was given, no natural resistance was found to exist in guinea pigs, thus confirming the observation of the investigators of the British Commission, and others.

Site of Inoculation.

Our experience with inoculation of the virus in sites other than the hairless pads of guinea pigs may yield some information concerning epizootics. After a proper injection in the pads, all or over two thousand guinea pigs showed the typical experimental disease within one to two days. In those, however, which were injected intramuscularly, intraperitoneally, subcutaneously, or intracutaneously in the abdominal skin, from 20 to 65 per cent failed to react with demonstrable lesions. In the guinea pigs which did yield typical signs, but only in the pads or in the tongue, the latter were very mild and were noted for two to six days after inoculation. In none were changes seen at the site of injection. The indication is that the virus is peculiarly epitheliotropic and probably has a limited portal of entry into the animal's body.

Titration of Immune Serum.

In view of the fact that immune serum, even in amounts of 10 cc., fails to prevent the primary lesions in guinea pigs, the protective value of such serum is measured by the quantity needed to prevent secondary lesions. Serum from normal cattle, swine, horses, rabbits, or guinea pigs fails, in any case, to prevent the generalization of the disease. The serum titration as practiced at present is not altogether satisfactory, and is due to the failure to regulate the infecting dose and inability to prevent the constant primary lesions. It appears that comparative serum titrations, employing graduated doses of virus, injected intradermally in sites other than pads, subcutaneously, intramuscularly, or intraperitoneally, may yield more satisfactory results, for by these methods the inoculation, or primary lesion is suppressed, and the infecting dose definitely regulated.

Artificial Hyperimmunization.

Attempts to hyperimmunize cattle and a horse by repeated injections of virus failed to produce more potent serum than that of convalescent animals. Indeed, serum from this horse was absolutely of no protective value. That from hyperimmunized guinea pigs and rabbits yielded the greatest protection (0.01 to 0.1 cc., neutralized the average infecting dose against generalization of the disease). In addition, comparison with hyperimmune serum obtained elsewhere (two samples from Riems-Loeffler type) revealed no greater protective value in the latter than some sera from recovered foot-and-mouth disease cases—either our own material, or that sent us from Denmark or Sweden. To summarize our experiments on this phase of the problem, it appears that the rabbit (or the guinea pig) can be hyperimmunized more successfully than the other species, and thus furnishes a means for further studies.

Immunity After Artificial Immunization.

In a limited study, we found that following injections of immune cattle serum, plus virus, if guinea pigs failed to yield secondary lesions they lost their resistance more rapidly than those animals which had passed through the experimental disease, or than those which showed secondary lesions even when immune serum was employed. Furthermore, immune serum plus virus injections in varying proportions, allowed to be in contact from one to several hours, either induced the disease in some instances or failed to protect in others. Finally, single or repeated inoculations of avirulent blood, that is blood containing active virus, kept for forty-eight hours at 37°C., did not protect guinea pigs. If we make any conclusions from our experience, we should state that avirulent material does not induce immunity, and there is no method devised to the present by which attenuated or active virus can be employed for immunization without first causing manifest lesions.

Susceptibility of Horses.

Five horses inoculated with foot-and-mouth disease virus, both "O" and "A" types, by scarification of the mucous membrane of the

mouth, failed to show the disease. In addition, a horse injected intramuscularly also failed to do so. The horse is evidently not susceptible.

Intradermal Diagnostic Tests.—Employing various antigens, no practical intradermal diagnostic test was revealed either in guinea pigs or cows. We found, however, a slight sensitiveness of the skin to the antigen in some animals at the height of the disease.

Complement-Fixation Tests.—Employing different antigens and immune or hyperimmune serum from cattle, a horse, hogs, guinea pigs, and rabbits, we were unable to disclose any complement-fixing bodies.

Comparative Studies of Vesicular Stomatitis and Foot-and-Mouth Disease.

The similarity of the clinical picture of vesicular stomatitis and of foot-and-mouth disease in cattle is so strikingly close that, in the interests of the spread and control of epizootics, we devoted considerable time to a study of the comparison of the two conditions. We found that vesicular stomatitis can be readily transferred to guinea pigs, to cattle, and to swine. In these animals, while there may be at times certain clinical differences, there is generally great difficulty in distinguishing the natural or the experimental disease from foot-and-mouth disease induced by one or another strain of the virus of the latter. In these animals, however, no evidence was detected of cross-immunity between vesicular stomatitis and foot-and-mouth disease virus, either "O" or "A" type. Horses, however, are resistant to foot-and-mouth disease, but are quite susceptible to vesicular stomatitis infection. Hence this animal can be regarded as the best test animal for a differential diagnosis. For further details of other methods of differentiating the two viruses, the article in the *Journal of the American Medical Veterinary Association* (*loc. cit.*) should be consulted.

We have also found that the virus of vesicular stomatitis is filterable through Berkefeld V and N candles, through Seitz' asbestos discs, and through Chamberland bougies, sizes L3 and L7. As is the case with the virus of foot-and-mouth disease, to be mentioned later, the virus of vesicular stomatitis is not filterable under ordinary condi-

tions through Chamberland L11 type bougies, and shows the same tendency to absorption in the walls of denser electronegatively charged filters.

Physical and Chemical Properties of Foot-and-Mouth Disease Virus.

The Incitant.—There is no conformitory evidence that the various kinds of bacteria of the ordinary species which have been advanced to the present as the inciting agents of the disease, actually play this part. It is now universally accepted that the incitant is a filter-passer which has eluded artificial cultivation by others and ourselves. The most promising efforts in the direction of culture were those of Frosch and Dahmen, but the results of the German, British, this American Commission, and others could not confirm their findings. Hence at this moment, the casual agent may be regarded as non-cultivable by means now available.

Titration of the Active Agent.—We have found that the active agent can induce the experimental disease in dilutions of 1:10,000,000, and that generally the period of incubation and the severity of the experimentally induced disease are proportional to the concentration of the incitant. The marked activity of the virus, as manifested by pathogenicity in very high dilutions, has, therefore, an obvious connection with the difficulty of controlling epizootics, and may indicate the minuteness of size of the incitant. We have delimited its relative size, by a series of "molecular" filtration tests, to 20 to 100 mμ in diameter, and by similar means and by cataphoresis experiments we have demonstrated that the virus may be particulate. We cannot subscribe to the opinion that the virus is of a fluid character, of a *contagium vivum fluidum.*

Cataphoresis.—The electric charge carried by the active agent is positive, as determined by cataphoresis. Its isoelectric point is at pH = about 8. This explains, among other phenomenon, certain filtration reactions to be described.

Centrifugalization.—The virus could not be sedimented at 2,500 to 3,000 r.p.m. for two hours. Nor did we succeed in removing, by this method, the so-called "inhibiting" bodies of Frosch and Dahmen. The inability to depose the virus supports our other experimental findings on its minuteness.

Filtration.—We found the virus to be regularly filterable through Seitz' asbestos discs and through Berkefeld V candles. In Berkefeld N candles, however, there was some adsorption of the electropositive virus in the electronegative filters. Through Chamberland bougies, sizes L1 to L5, the virus passed regularly; but in the denser bougies, L7 and L9, only occasionally and in the still more dense L11 filter, it was completely retained. Here again adsorption was due to oppositely charged materials. Through L11 Chamberland bougies, however, the virus passed freely when the charge of the latter was changed to negative.

Supporting the conclusion of the British Commission, we failed to obtain active filtrates through various types, thicknesses, and densities of collodion membranes, which are also electronegative. We did, however, obtain positive filtrates with Bechold's ultrafilters, but then only with the most porous of the series.

Chemical Properties.—The minuteness of size and the electropositive charge indicate the possibility of the virus entering into firm combination with proteins, ordinarily electronegative, or its capability of being protected by larger collodial agglomerations. Indeed, we have shown that the protective action is quite marked and explains the abnormal resistance which the virus shows to such antiseptics as alcohol, acetone, bichloride of mercury, cresol, etc. Sixty per cent alcohol, for example, kills staphylococci in one minute, but not the virus until after at least twenty-six hours.

In laboratory tests these antiseptics are added to pure cultures of ordinary bacteria. The action is then direct. In the case of the virus, on the other hand, the active agent is non-cultivable and is therefore admixed with tissue or exudate. The chemicals, in consequence, cause a more or less heavy coagulation of proteins. These coagula protect the virus. If coagulation is prevented, as we have done, and the virus is placed directly in contact with the reagent, then it is more sensitive to the destructive effects of the chemicals than are staphylococci. As a corollary, such reagents as sodium hydrate or antiformin (which is prepared with alkali) that do not coagulate proteins, are highly virucidal. Sodium hydrate 1 to 2 per cent, or antiformin 1 per cent, can kill the virus within one minute.

Cultivation Experiments.

No multiplication of the virus *in vitro* was observed. We have found, however, that the optimum conditions necessary for the preservation of the virus in artificial media are as follows:

The hydrogen ion concentration of the medium should be 7.5 to 7.6, not only at the beginning, but, and more important, at the conclusion of the period of observation. A strict anaerobic atmosphere is also favorable, as is a temperature below 37°C. A semisolid structure of the medium appears to be advantageous and this can be effected by the use of 0.25 per cent agar, or 10 per cent gelatin. Of the two, gelatin is more desirable, and of the latter the most effective preparation is gelatin prepared after the manner of Loeb and adjusted to the proper hydrogen ion concentration with potassium hydroxid. In this medium the virus survived longer than 69, but shorter than 100 days. Gelatin is the simplest of protein media available and its use is in keeping with the principle that the virus requires only a simple material for life: the addition of organic substances or of proteins caused destruction of the active agent.

From the standpoint of technic, it was found necessary, when comparing two or more media for their effectiveness, to employ all of them in a parallel experiment with the same sample of virus, for the factors of potency of the active agent, contamination, and changes in hydrogen ion concentration, if variable, may give rise to faulty interpretations. Furthermore, activity in three successive subplants may be regarded as mere preservation but not multiplication of the virus.

We have consistently failed to confirm the results of Frosch and Dahmen in respect to their reported artificial cultivation of the active agent. In this failure of corroboration, as well as in many other experimental results, we are in complete accord with the workers of the British Commission, and others.

Finally, in our forthcoming report, we shall discuss the fact that nothing has as yet been presented to show that the virus of foot-and-mouth disease is of an inanimate character.

COMPARATIVE PATHOLOGY OF SOUTH AFRICAN JAGZIEKTE AND MONTANA PROGRESSIVE PNEUMONIA OF SHEEP.

BY E. V. COWDRY AND HADLEIGH MARSH.

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PLATES 16 TO 18.

Recent studies on a disease of sheep occurring in the northwestern United States, called "progressive pneumonia,"[1,2] appear to have a very definite bearing upon our conception of a South African disease called jagziekte, and *vice versa*. The two conditions have been studied independently, the investigators in each case considering the disease as unique and peculiar to their respective geographical localities. Comparison of publications on the two diseases has brought to our attention the fact that these two chronic pneumonias of sheep, occurring in widely separated parts of the world, are very similar, and apparently are identical in the fundamental characteristics which differentiate them from other described forms of pneumonia. It therefore seems profitable to correlate our knowledge of two such closely related diseases by making a comparative study of the lesions, particularly as in both cases, the diseases cause a considerable loss to the sheep industry, and in neither case has the etiology been definitely established.

Jagziekte has been known in South Africa since 1893. The term is derived from the Dutch *jagt*, to drive, and *ziekte*, a sickness, and is intended to call to mind the fact that the first symptoms appear in animals which are fatigued as a result of having been driven for some distance. The principal lesions are pneumonic and proliferative in character. We have shown the tumor-like proliferations of pulmonary epithelium to several investigators very familiar with cancer. Some

[1] Marsh, H., *J. Am. Vet. Med. Assn.*, 1922–23, lxii, 458.

[2] Marsh, H., *J. Am. Vet. Med. Assn.*, 1923–24, lxiv, 304.

do not hesitate to return a diagnosis of true cancer, while others are more conservative and remark, that, if the same conditions were met with in the human lung, they would classify them as cancerous. No cases have been known to recover. The disease is certainly contagious and is so greatly feared that the farmers immediately kill off all contacts, although they may appear to be entirely healthy.[3]

"Progressive pneumonia" of sheep has been studied in Montana since 1915. Sheep affected with this disease are called "lungers" by the sheepmen. Many sheepmen state that they have known the condition for the last 30 years, but it has apparently increased in recent years and invaded new territory. This is apparently a disease of range sheep as opposed to farm flocks. It is believed that the disease is infectious, and that the infection is probably secondary to mechanical causes. The lesions are similar to those of jagziekte and the prospect of recovery is just as hopeless.

The present study was accordingly commenced in the hope that a detailed comparison of the two conditions might reveal points of correspondence or of divergence, which have hitherto been overlooked, because they have never before been considered together; and, further, that this new information might in turn be of service in unraveling the obscure etiology of both diseases.

Material.

The material upon which this paper is based consists of tissues collected in South Africa from 33 sheep suffering from jagziekte and from 72 control healthy sheep (including some American sheep employed as geographic controls). In addition many clinical cases of jagziekte were observed. The progressive pneumonia material consists of tissues from 46 cases and from 18 supposedly normal sheep. Both histological and bacteriological examinations were made in progressive pneumonia and the symptoms of many typical cases were noted.

OBSERVATIONS.

Before reporting our studies on the comparative pathology of jagziekte and progressive pneumonia, the accompanying table is submitted in order to give a general outline of the two conditions.

Specific reference may be made to one statement in the table; namely, that enclosures in which sheep suffering from jagziekte have been kept constitute sources of infection for incoming healthy sheep, which apparently does not hold

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[3] Mitchell, D. T., *3rd and 4th Rep. Director Vet. Education and Research* (Sir Arnold Theiler), Union of South Africa, 1915, 585.

TABLE I.

	Jagziekte	Progressive pneumonia
Epidemiology	1. Average mortality in infected areas 1.6 per cent *per annum*	1. About 2 per cent *per annum* in infected bands
	2. Occurs throughout the year	2. The same
	3. All ages affected, but chiefly at 3 yrs.; never lambs	3. Most of the deaths occur in sheep 4 yrs. old and over; but many clinical cases have been observed in 2 yr. olds, a few in yearlings, and early stages have been seen in lambs on postmortem examination
	4. Occurs in both range sheep and farm flocks, but chiefly the former	4. Occurs in range sheep, but not in farm flocks
	5. High altitude and low humidity	5. The same
	6. High mean annual temperature	6. Lower mean annual temperature
	7. No evidence that the character of water supply and of the grazing are determinative factors	7. The same
	8. Apparently transmitted by contact, since it appears after importation of new sheep	8. The same
	9. Enclosures in which cases of jagziekte have been kept are said to constitute sources of infection for healthy sheep for 10 days or more after the diseased sheep have been removed	9. Not known to be the case
Symptoms	1. Increasing respiratory distress with catarrhal nasal discharge	1. Increasing respiratory distress, with but little nasal discharge in most cases
	2. Extreme emaciation	2. The same
	3. Little or no increase in temperature. Failure to note a temperature reaction may be due to the fact that the sheep are seldom observed clinically in the earliest stages of the disease, which are in fact not detectable	3. The same

TABLE I—*Continued.*

	Jagziekte	Progressive pneumonia
Symptoms— *continued*	4. The disease extends over a period of several mos. At the outset destructive lesions may exist which are not revealed by clinical symptoms	4. The disease extends over a period of several mos. after symptoms appear. The period during which it exists, but cannot be detected clinically, may extend for several mos. or even yrs.
Prognosis	Duration of life may be prolonged by careful treatment, but it is said to be invariably fatal.[3] There is, however, some evidence that animals in the earliest stages may recover[4]	The same
Prophylaxis	Immediate slaughter of all contacts and the abandonment or careful cleaning of quarters occupied by sick sheep	Slaughter of all recognizable cases
Etiology	1. No organisms have thus far been constantly observed in the specific lesions, although bacteria of several kinds are frequently present, usually in association with inhaled dust-like foreign material	1. Two bacteria have been constantly isolated in cultures made from the lungs, one or both of which may be the causative agents[2]
	2. Eosinophil leucocytes, which one would expect in nematode infestations, are not unusually abundant	2. The same
	3. Poor general condition and insufficient food are not of primary importance as predisposing factors	3. The same
	4. It has never been possible to transmit the disease by the inoculation of material of any kind from diseased animals	4. Repeated intratracheal inoculations of a diphtheroid from affected sheep produced small lesions of the same character as the extensive lesions found in naturally diseased animals. One sheep which was inoculated intratracheally with lung tissue developed the disease

TABLE I—*Concluded*.

	Jagziekte	Progressive pneumonia
Etiology— *continued*	5. The intratesticular and intra-cerebral inoculation of tissue—procedures indicated by the tumor-like appearance of the epithelial growth—have not been attempted	5. The same
Pathology	1. The specific lesions are restricted to the thoracic cavity	1. The same
	2. The primary changes are found in the interalveolar tissue of the lungs. They occur in foci several mm. in diameter and consist of engorgement of the alveolar capillaries and of the interstitial accumulation of macrophages and lymphocytes. Frequently they are associated topographically with deposits of above mentioned foreign material[4]	2. The same, except that this primary interalveolar infiltration seems to be more extensive
	3. Exudation of macrophages and of leucocytes then takes place into the alveoli resulting in a typical chronic catarrhal pneumonia. At the same time there is a marked and very luxuriant tumor-like proliferation of the epithelial cells of the alveoli and bronchioles[5]	3. The same, except that the epithelial proliferation is usually less pronounced and resembles more a benign hyperplasia. The growth is distinctly less invasive and less neoplastic in appearance. Dividing epithelial cells are more abundant, also alveolar casts
	4. There is sometimes a slight tendency toward localized resolution of the pneumonic process, but this is rare and never takes place on a large scale	4. Not observed
	5. The blood supply is reduced and extensive fibrosis takes place. The animals apparently die from asphyxiation through reduction in respiratory area	5. The same

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in progressive pneumonia.   It is based upon casual observation only and is diffi-
cult to prove, because it has been found that jagziekte, in its earliest stages, is not
recognizable clinically[3],[4] so that supposedly healthy sheep employed in the ex-
periments may not be in reality free from the disease to begin with.

Examination of gross specimens shows that the lesions commence in
definite foci and spread thence throughout the pulmonary tissue.   In
the more central parts of the affected areas the lesions are older, and
fibrosis and consolidation have frequently set in.   It is possible,
therefore, by selecting specimens for histological examination, first
remote from the principal lesions and then nearer and nearer to them,
to secure a series of preparations which give some idea of the sequence
of changes involved.   The earliest changes may, we believe, occa-
sionally also be found by the examination of sheep which have as yet
shown no clinical signs of the disease, but which come from affected
flocks, through a comparison of their tissues with those of sheep known
to be free from both diseases.[4]   Lungs from cases of jagziekte of this
kind show the infiltrative, exudative and slightly proliferative changes
which we are about to describe, uncomplicated by the pneumonia and
fibrosis characteristic of more advanced stages.

In the plates illustrating the lesions, the photomicrographs on the
left are from cases of jagziekte and those on the right from cases of
progressive pneumonia.   For additional illustrations of the diseases
considered separately, reference may be made to earlier publications.[1]–[5]

The Interalveolar Tissue.

In both diseases the primary changes center in the interalveolar
tissue, which is thickened (Figs. 1 and 2).   They are identical in
quality but seem to be rather more extensive in progressive pneumonia.

The thickenings are caused principally by accumulations of lympho-
cytes and large mononuclear cells which have been variously called
macrophages (Metchnikoff), endothelial leucocytes (Mallory) and
polyblasts (Maximow).   None of them exhibits signs of mitotic
division.   Sometimes, in both conditions, the lymphocytes may
greatly outnumber the large mononuclears and give rise to nodules

[4] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 323.
[5] Cowdry, E. V., *J. Exp. Med.*, 1925, xlii, 335.

recognizable in gross specimens. The nodules are often peribronchial in position. Both of these cells are accompanied by only a few polymorphonuclear leucocytes. Eosinophil leucocytes are not unusually abundant either in the lesions or in the circulating blood.

The exact source of these large mononuclears is difficult to ascertain, although they are probably chiefly of hematogenous origin. No evidence was found that they are produced through the multiplication of the endothelial cells of the alveolar capillaries as claimed by Permar.[6] In some jagziekte preparations they were seen within the capillary lumina in contact with erythrocytes and free from the lining endothelium (foot-note 4, Fig. 9). In all likelihood they may also arise from cells resident in the interalveolar tissue; in other words, from the rhagiocrine cells of Renaut, or the histiocytes of Kiyono (to employ only a few of the available synonyms). That they may be derived from the lymphocytes which are likewise present normally in small numbers in the interalveolar tissue is to be considered as a third possibility in view of Maximow's[7] observation that freshly emigrated lymphocytes are capable of transformation into large phagocytic ameboid cells; that is to say, into the mononuclear cells. The lymphocytes, in turn, may emigrate from the alveolar capillaries, since, although we have no specific information regarding the pulmonary lymphatics of sheep, Miller[8] was unable to find in man any lymphatics in the walls of the air sacs beyond the ductuli alveolares.

The thickenings in jagziekte frequently also contain, in addition to the cells enumerated, deposits of inhaled foreign material which have been already reported,[4] and there is reason to believe that in progressive pneumonia substances of this kind may likewise act as predisposing factors.

With the foreign material in jagziekte, bacteria of several kinds are frequently noted on microscopic examination, and it is probable that cultural methods would bring to light the existence of a varied bacterial flora. Several bacterial invaders are probably also concerned in progressive pneumonia, the bacteriology of which has been much more systematically studied.[2]

The fact that Marsh succeeded in experimentally producing small lesions, like those of typical cases of progressive pneumonia, by the intratracheal inoculation of a diphtheroid isolated from affected sheep

[6] Permar, H. H., *J. Med. Research.*, 1920–21, xlii, 147, Plate IV.

[7] Maximow, A. A. *Physiol. Rev.*, 1924, iv, 533.

[8] Miller, W. S., *Am. Rev. Tuberc.*, 1919–20, iii, 193.

is suggestive, especially in view of some recent experiments by Grumbach,[9] who, by the injection, similarly of a diphtheroid ("*Coryne-bacillus diphtheroide*"), caused pulmonary lesions in guinea pigs, which he regards as almost identical with those of jagziekte in sheep. Grumbach has very courteously sent microscopic preparations to us and we have verified the close resemblance which some of them bear to jagziekte and progressive pneumonia.

Coincident with these interalveolar infiltrations in both diseases, there is often a noticeable localized vascular engorgement.

The Alveolar Exudate.

This consists chiefly of large mononuclear cells which are like those already mentioned in the interalveolar tissue but are modified to some extent owing to enhanced phagocytic activity. Their appearance is the same in progressive pneumonia as in jagziekte in which they have been described in detail in a previous paper[4] which is supplemented by several photomicrographs. Attention may however be directed to their properties, as represented in the lower right hand corner of Fig. 8. There may be some polymorphonuclear leucocytes in addition, but the latter do not as a rule predominate in either jagziekte or progressive pneumonia except in later stages, when an acute pneumonic process is often met with (Figs. 11 and 12). Polynuclear giant cells, arising from the mononuclears, are often encountered. They are illustrated in Fig. 11 among the leucocytes. There is some desquamation of the epithelial cells. Coagulated blood protein is not abundant in the exudate.

The nature of the mononuclear cells, which are frequently known as "epithelioid cells" and "dust cells," is difficult to determine. Some investigators[10–13] believe them to be epithelial cells which have hypertrophied and desquamated into the alveolar lumina. Others incline to the view that they are of hematogenous origin. Permar[6] stimulated the production of these cells by injecting into the air spaces finely divided foreign material and followed all stages in their migration, having

[9] Grumbach, A., *Bull. Assn. franç. étude cancer*, 1926, xv, 213.

[10] Briscoe, J. C., *J. Path. and Bact.*, 1908, xii, 66.

[11] Sewell, W. T., *J. Path. and Bact.*, 1918–19, xxii, 40.

[12] M'Fadyean, Sir John, *J. Comp. Path. and Therap.*, 1920, xxxiii, pt. 1, 1.

[13] Mavrogordato, A., *J. Hyg.*, 1918, xvii, 439.

first marked them by vital staining with pyrrhol blue. He found no evidence that the nucleated or non-nucleated respiratory epithelial cells, described by Ogawa,[14] took up the dye. Lewis, Willis and Lewis[15] closely correlated the types of cells observed in experimental pulmonary tuberculosis as seen in sections and with the aid of supravital stains. They discovered that the epithelioid cells, both in the tubercles and within the lumen of the alveoli, resembled the large mononuclears (macrophages or monocytes) of the blood in many details of their structure (nuclei, centrosomes, mitochondria, neutral red granules) as well as in their phagocytic properties.

In jagziekte we believe that these large mononuclear cells may in the vast majority of cases be distinguished from desquamated respiratory epithelial cells by the following criteria:

1. Their nuclei are often kidney-shaped (and may thus be contrasted with the spherical nuclei of the epithelial cells) and by staining with iron-hematoxylin a centrosome, or a diplosome, is revealed on the side of the nuclear concavity.

2. The centrosome is always centrally placed (remote from the periphery of the cell) and the cytoplasmic granules about it are arranged radially. In hematoxylin and eosin preparations the position of the centrosome is marked by an area of cytoplasm which stains less intensely. Methods for the supravital staining of blood elements[16, 17] were not employed and it is doubtful whether they would be helpful in this case, because the question of the source of the cells thus examined (*i.e.* from the alveolar lumina, or ruptured alveolar capillaries, or interalveolar tissue) would remain.

3. There are many rod-like mitochondria in the cytoplasm accompanied by droplets of fat which may be colored with Sudan III. Both of these components are difficult to find in unaltered epithelial cells.

4. The mononuclear cells are often present in such enormous numbers as to suggest some almost inexhaustible reservoir of origin, such as the blood stream, as contrasted with the limited surface of the alveolar walls.

5. They are actively phagocytic for just those substances which macrophages are known to take up, and, on general principles, it would seem unlikely that desquamated epithelial cells would exhibit this property. In all other parts of the body desquamating epithelial cells are either dead or dying.

6. They appear within the alveoli before the epithelial cells hypertrophy and multiply *in situ*, or extend over the internal alveolar surface from restricted clusters, if we accept Ogawa's conclusion regarding their normal distribution.

7. What appear to be actual stages in the entry of these mononuclears into the

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[14] Ogawa, C., *Am. J. Anat.*, 1920, xxvii, 333.

[15] Lewis, M. R., Willis, H. S., and Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1925, xxxvi, 175.

[16] Cowdry, E. V., *Internat. Monatschr. Anat. u. Physiol.*, 1913–14, xxxi, 267.

[17] Simpson, M. E., *Univ. Calif. Pub. Anat.*, 1921, i, 1.

alveolar lumina, may in rare instances be seen and identified by their position and the hour-glass-like shape of their nuclei, when they are fixed half inside and half outside the wall of the alveolus (foot-note 4, Fig. 10).

8. In more advanced stages of the disease, when the epithelial proliferation has become extensive, so that all the alveoli are lined by cubical or columnar cells, the number of large mononuclears within the alveolar lumina is markedly diminished (see Figs. 5 and 6). This decrease in the mononuclears as far as can be determined is coincident, or follows immediately after, a constriction of the blood vessels caused by the pressure of the proliferating epithelial tissue and by inter-alveolar fibrosis. If the mononuclear cells were produced, for the most part, by the desquamation of epithelial cells, one would expect them to appear when the epithelial cells are most abundant instead of at a time when few if any of them may be seen and none of them has undergone any noticeable preparatory hypertrophy.

9. Some epithelial cells do, however, unquestionably desquamate. They break away from the alveolar wall in groups, seldom singly, by the operation of some factor or factors which inhibit or destroy their cohesive properties. Frequently the entire hypertrophied layer of alveolar epithelium splits away from the wall of the alveolus and comes to occupy a position within it as indicated in Fig. 7 of jagziekte and in Fig. 8 of progressive pneumonia.

The origin of most of the mononuclear cells seems, thus, to be the same—namely, from the contents of the alveolar capillaries and the interalveolar tissue—in both jagziekte and progressive pneumonia, although we have not examined the centrosomes, mitochondria and neutral fat in the latter condition.

The Epithelial Proliferations.

There is some question whether distinctive proliferations of the respiratory epithelium are to be observed in all cases. While Theiler[18] considers them to be definitely indicative of jagziekte, Mitchell[3] failed to find them in two out of fifteen cases and did not refer either to their presence or absence in three others. In progressive pneumonia Marsh has not always found them. In general they seem to be of slightly more constant occurrence in jagziekte.

In both conditions they commence in isolated foci like those represented in Figs. 2 and 3. That they originate through a metamorphosis of alveolar epithelium may be readily ascertained by the study of serial sections, which shows that they are often entirely separate and apart from the epithelium of the bronchioles. But the bronchiolar

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[18] Theiler, Sir Arnold, personal communication.

epithelium also proliferates, though less extensively. These foci of overgrowth of the alveolar epithelium later merge with the result that large masses of tissue become almost (or in fact) adenomatous (Figs. 5 and 6) and present an appearance resembling superficially a mammary gland regenerating during lactation. The cells are sometimes, in the older growths, quite atypical in their properties (footnote 5, Fig. 12). They are generally arranged in a single layer but in both diseases many of them may become superposed so that irregular masses of cells result. Within the bronchiolar proliferations dense and circumscribed clumps of polymorphonuclear leucocytes, each from about ten to twenty cells in number, may occasionally be distinguished.

Mitotic figures are of comparatively rare occurrence but appear to be a little more numerous in progressive pneumonia. Nuclear appearances indicative of widespread amitotic multiplication of the epithelial cells were seldom observed in either disease.

In general, the proliferations seem to be somewhat more luxuriant and tumor-like in jagziekte, but cases of progressive pneumonia are often observed in which they are equally highly developed and constitute just as conspicuous features of the disease. They are slightly invasive in both (foot-note 5, Fig. 11), but never metastasize, even to lymph glands within the thorax. Other parts of the body are not affected except by the general cachexia.

The proliferations are obviously secondary to the infiltrative and exudative changes already referred to. In common with hyperplasias of respiratory epithelium in man[19,20] they constitute a sort of aftermath of a preexisting infection. But they are undoubtedly more marked than any which have been reported in man, except in the case of definite neoplasms in which, however, the distinctive and primary interalveolar changes, already referred to, are generally absent.

Other Lesions.

A comparison of Fig. 7 of jagziekte and Fig. 8 of progressive pneumonia with Figs. 5 and 6 of earlier lesions will illustrate the commenc-

[19] Winternitz, M. C., Mason, I. M., and McNamara, F. P., The pathology of influenza, New Haven, 1920.

[20] Hart, C., *Deutsch. Arch. klin. Med.*, 1904, lxxix, 108 (measles).

ing interalveolar fibrosis which becomes very marked in advanced stages of both diseases and leads to widespread consolidation. The occurrence of small definitely outlined areas of myxomatous tissue is also typical (Figs. 9 and 10).

The pneumonic process is, as far as we can ascertain, of fundamentally similar nature in jagziekte and progressive pneumonia. Corresponding parts of the lungs are affected and it spreads in the same way, bringing about eventually the death of the animal.

Comparison of Jagziekte and Progressive Pneumonia with Verminous Pneumonia.

Through the kindness of Sir John M'Fadyean we have had the privilege of examining also a specimen from a case of verminous pneumonia in sheep. On the basis of this specimen and of his published description[12] it would apparently be difficult, through microscopic examination alone, to distinguish the lesions of this disease from those of jagziekte, unless perhaps very abundant material were available. Like jagziekte, the lesions of verminous pneumonia differ from those of progressive pneumonia chiefly by somewhat lesser involvement of the interalveolar tissue and by slightly more intense epithelial proliferation. The presence of nematodes and of their ova within the lungs in verminous pneumonia and their undoubted rôle in the production of the disease, as discovered by M'Fadyean, led us to make a very careful search for animal parasites in both jagziekte and progressive pneumonia, which was, however, unavailing.

DISCUSSION.

The pathogenesis of the lesions which we have briefly described in jagziekte and progressive pneumonia suggests the conclusion that the lungs have been subjected over a rather long period of time (4 to 8 months) to a variety of injurious influences, mechanical, in the form of foreign material, and infective, through bacterial invasion. It seems indeed surprising that similar conditions have not been reported from still other localities. With the possibility in mind that, although such accounts have not been published, the diseases may nevertheless occur, letters of inquiry were sent to Professor José Lignieres at Buenos Aires (Argentina) and to Professor Harold A. Woodruff at Melbourne (Australia). The answers received, together with a letter from Dr. H. R. Seddon of the Department of Agriculture, New South Wales, indicate that no cases of disease resembling jagziekte

or progressive pneumonia have thus far attracted attention in these great sheep-raising countries.

Definite predisposing influences are unknown, but that a dietary factor may nevertheless be involved is indicated by Theiler's study of jagziekte in horses,[21] which he found to be caused by the eating of a poisonous plant (*Crotalaria dura*), but as yet there are no observations available which point in this direction in the case of these two destructive diseases of sheep. Nor do we know just how closely jagziekte of horses resembles jagziekte of sheep. From Theiler's description it would appear that the epithelial proliferations are alike; but it is no longer possible to collect material for comparison without experimentally producing the disease by feeding; because as soon as its cause was discovered, preventive measures were taken which have completely banished it from the Union of South Africa.

In order to secure advice as to whether the action of some foreign protein might, in fact, constitute a specific predisposing factor in jagziekte, specimens were sent to Dr. C. F. Hoover of Western Reserve University. Dr. Hoover states that it is conceivable that repeated inhalations of protein may be responsible for such a chronic condition, but that if this were the case one would expect the attacks of respiratory distress to be paroxysmal, which is not characteristically so in either jagziekte or progressive pneumonia.

In regard to the precise cause of the epithelial proliferations, very little may be said. Robertson's[22] theory that they are produced by a malaria-like microorganism has received no support. It seems likely, when we take into consideration the occurrence of similar proliferations in verminous pneumonia,[12,23] and the multiple adenomata reported by Ebner,[24] that the respiratory epithelium of sheep is unusually prone to undergo this change. That epithelial proliferations are commonly produced by bacterial infections[25] and by a variety of experimental agencies[26] is well known. Yet as far as our

[21] Theiler, Sir Arnold, *7th and 8th Rep. Director Vet. Education and Research*, Union of South Africa, 1918, 59.

[22] Robertson, W., *J. Comp. Path. and Therap.*, 1904, xvii, 214.

[23] Hofman, V., *Časop. lék. česk.*, 1923, lxii, 65; abstracted in *J. Am. Med. Assn.*, 1923, lxxx, 1279.

[24] Ebner, A., *Z. Tiermed.*, 1899, iii, 161.

[25] Teutschlaender, *Centr. allg. Path. u. path. Anat.*, 1914, xxv, 424.

[26] Fischer, B., *Frankf. Z. Path.*, 1922, xxvii, 98.

present information concerning jagziekte is concerned it is still possible that the proliferations may arise through the action of a single ultravisible virus, as claimed by Theiler;[18] but we do not believe that the entire disease complex is caused by such a virus. It is interesting, in this connection, to recall that Roux[27] discovered the existence of an adenomatous proliferation of the respiratory epithelium in sheep-pox, but unfortunately he only referred to it very briefly and gave no illustrations.

If the proliferations in jagziekte and progressive pneumonia are in truth neoplastic, it is difficult to explain Mitchell's failure to transmit the disease by the inoculation of tissue fragments despite the fact that he did not avail himself of recent methods of intratesticular inoculation with a large series of animals. Perhaps the failure is due to the difficulty of producing experimentally the primary lesions, the nature of which we have stressed and which may be prerequisite to the growth of the tumor. Acceptance of the tumor hypothesis would render intelligible the fatal outcome in all recognizable cases of the diseases. Obviously, if we are dealing with a tumor, it does not mean that we are faced by one which of itself and independently spreads from diseased to healthy sheep; on the contrary, the view that we have to do with a neoplasm which comes in the wake of an acute infection or series of infections, most probably bacterial in nature, is not improbable.

SUMMARY.

As long as the exact etiology of jagziekte and of progressive pneumonia remains unknown, it cannot be said that they are identical, although on the basis of the observations detailed above, they certainly appear to be. It is extremely doubtful whether it is possible to distinguish between them. In the localities in which they occur, each is recognized as an infection quite distinct and apart from other better known pneumonias. Predisposing factors which lead to bacterial invasion are presumably of great importance in both. We have no precise information regarding the organisms concerned, but in both diseases, the initial changes are alike and occur in the interalveolar tissue and the subsequent proliferations of epithelium and the pneu-

[27] Roux, E., *Bull. Inst. Pasteur*, 1903, i, 49.

monia are of the same character and apparently follow in exactly the same sequence. Finally, in both diseases, there is much fibrosis and the animals inevitably die through depletion of respiratory area and pneumonia.

The view hitherto held, that jagziekte is a disease definitely restricted to the Union of South Africa, is thus rendered improbable. Nor does it appear likely that jagziekte is due to a single specific virus acting primarily upon the epithelial cells of the lungs, which is is likewise a conception widely accepted in South Africa.

EXPLANATION OF PLATES.

To facilitate comparison of the two diseases photomicrographs taken at a magnification of 240 diameters of approximately similar lesions are given in pairs, jagziekte on the left and progressive pneumonia on the right hand side.

PLATE 16.

FIG. 1. Interalveolar infiltration in jagziekte.

FIG. 2. The same in progressive pneumonia.

FIG. 3. Beginning epithelial proliferation in jagziekte.

FIG. 4. The same in progressive pneumonia with rather more interalveolar infiltration.

PLATE 17.

FIG. 5. More advanced proliferation in jagziekte.

FIG. 6. The same in progressive pneumonia.

FIG. 7. Desquamation of proliferated epithelium in jagziekte.

FIG. 8. Same in progressive pneumonia with less interalveolar change. The alveoli in the right lower corner contain typical large mononuclear cells.

PLATE 18.

FIG. 9. Nodule of myxomatous tissue in jagziekte.

FIG. 10. The same in progressive pneumonia. It will be noted that the cells are larger because they have not been shrunken so greatly by the fixative.

FIG. 11. Masses of polymorphonuclear leucocytes in the lumina of bronchioles in jagziekte. Many polynuclear giant cells are visible.

FIG. 12. The same in progressive pneumonia except that in this case the proliferated epithelium is rather lower, being more typical of the altered alveoli, and giant cells may be only indistinctly seen.
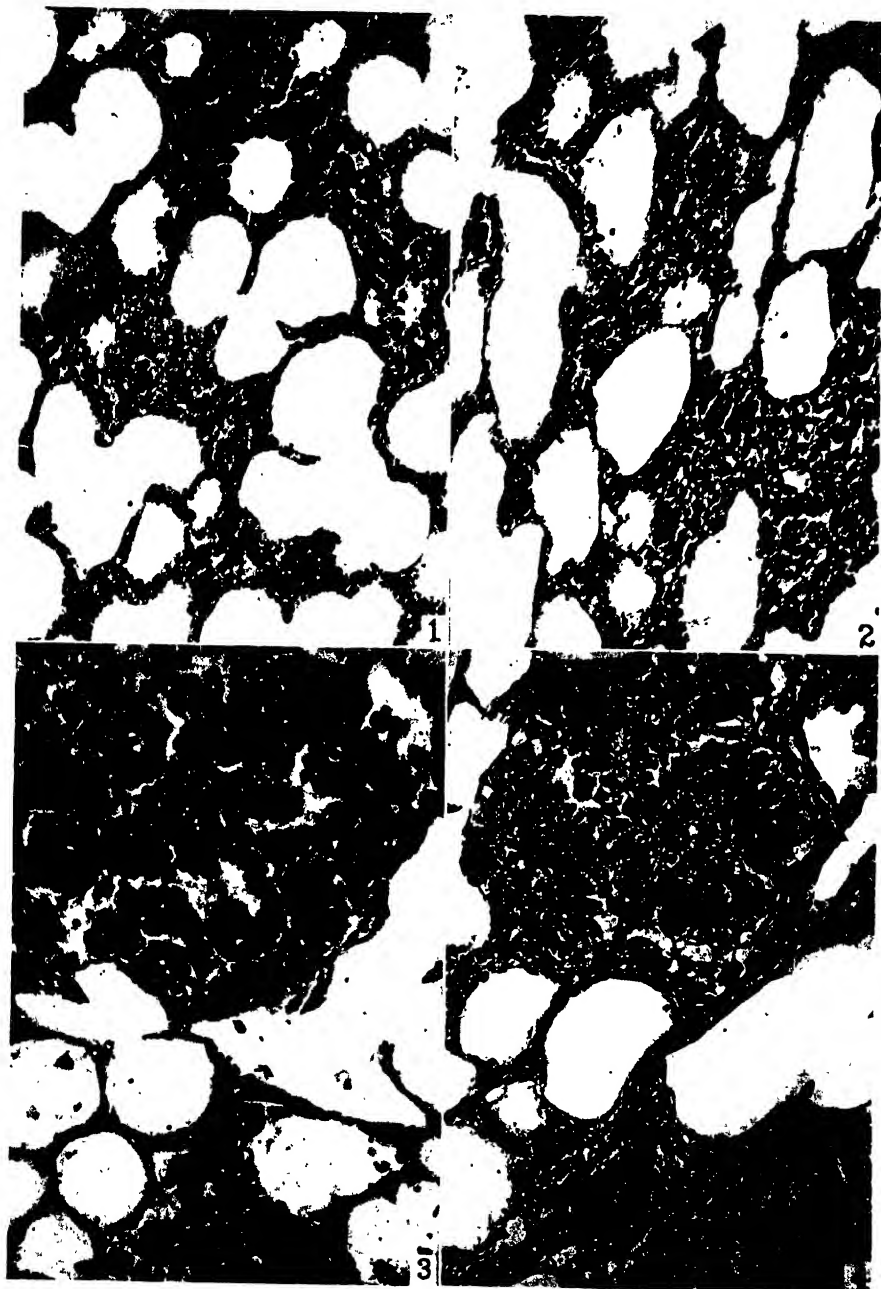
(Cowdry and Marsh: Jagziekte and pneumonia of sheep.)
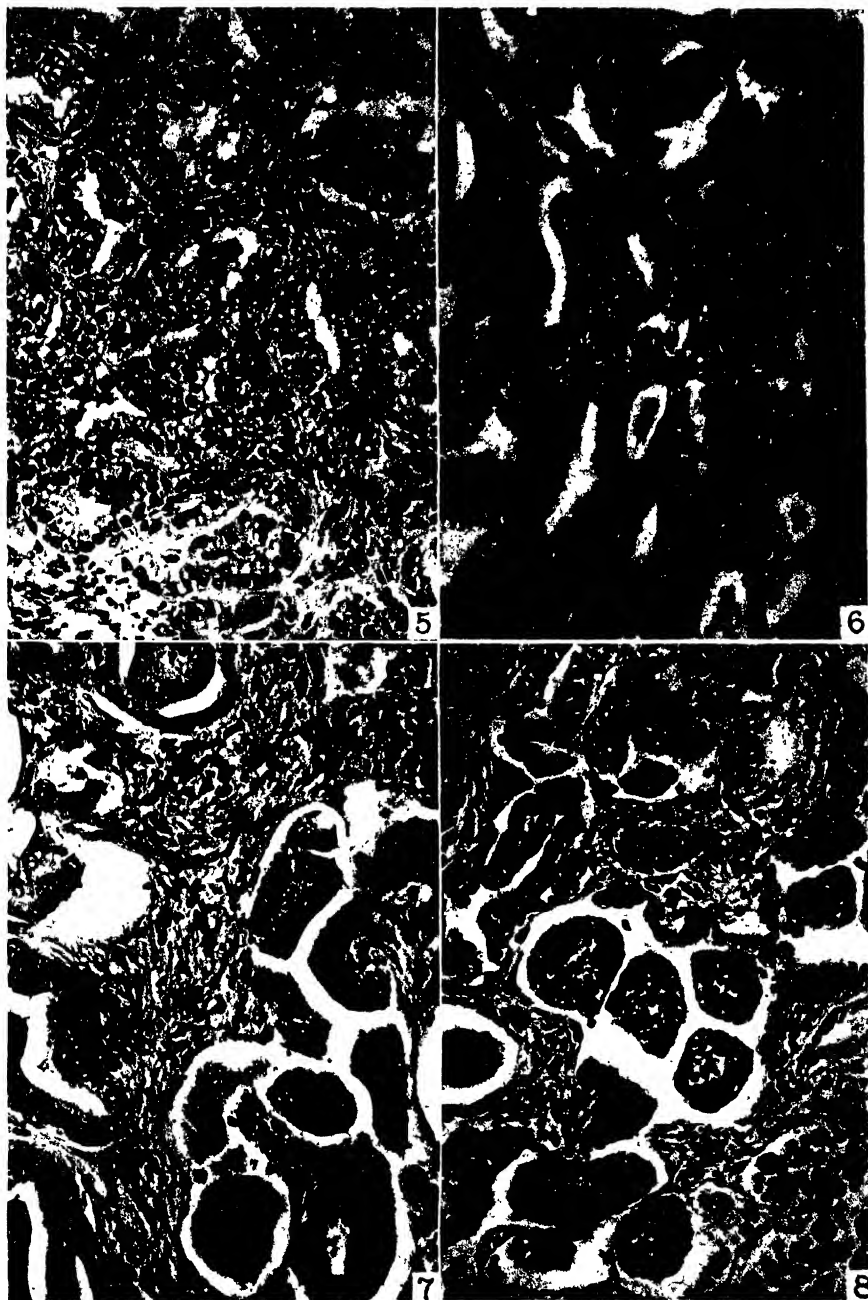
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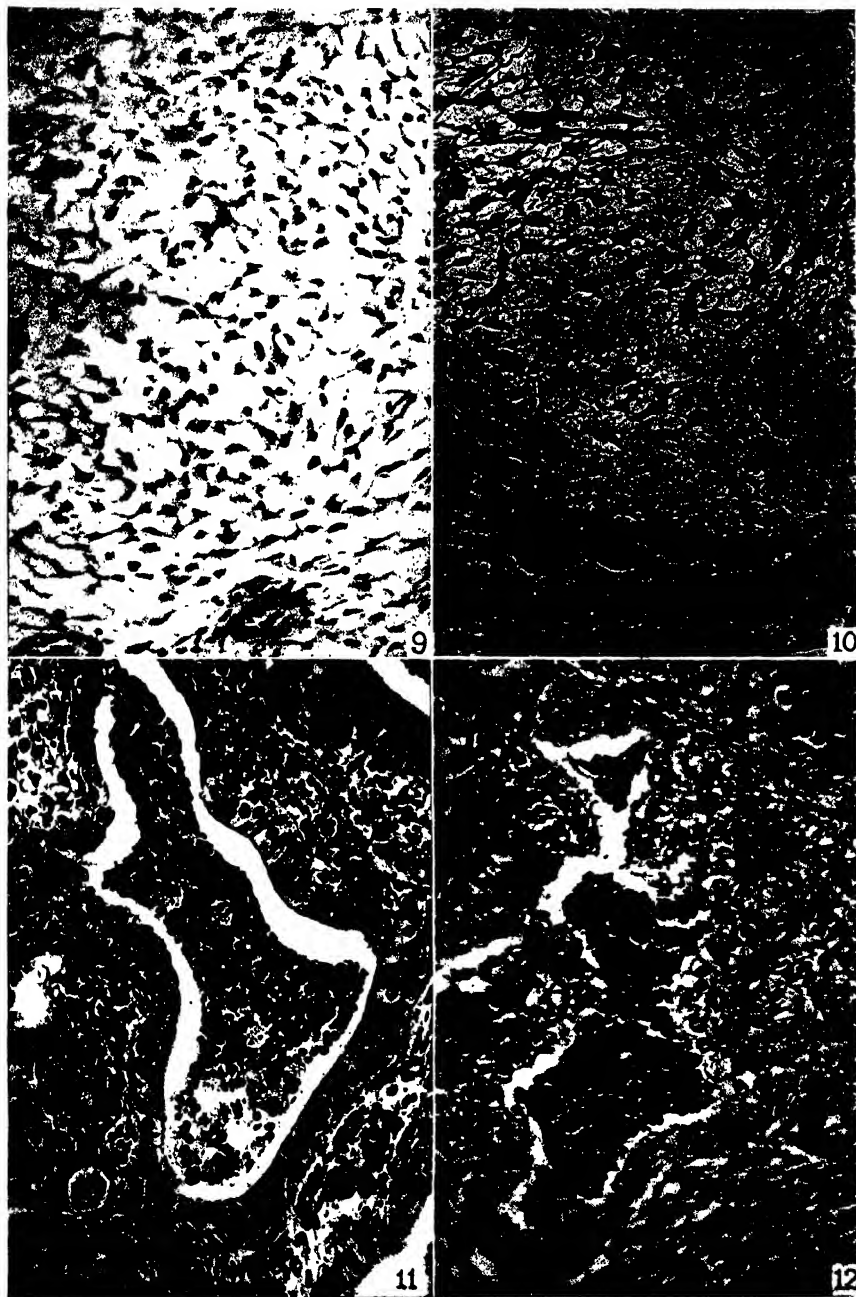
(Cowdry and Marsh: Jagziekte and pneumonia of sheep.)

INFLUENCE OF LIGHT ON THE GROWTH AND MALIGNANCY OF A TRANSPLANTABLE NEOPLASM OF THE RABBIT.

SECOND PAPER.

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The experiments reported in the present paper are a continuation of previous work dealing with the effect of light on the growth and malignancy of a transplantable epithelial neoplasm of the rabbit (1) and are a part of a general study on the relationship of external conditions to the physical constitution of normal rabbits and to the reaction of rabbits to certain experimental diseases.

The belief that there is a relationship between sunlight and the manifestations of this particular tumor arose, first, from the fact that a characteristic feature of the disease is the seasonal variability of its behavior and, second, that a correlation could be drawn between the prevailing level of sunshine and changes in the curve of sunlight, on the one hand, and variations in malignancy of the tumor process, on the other (2). In order to test this belief, experiments were carried out in which conditions of light could be controlled. The results of the first experiment (1) showed that, as compared with rabbits living under ordinary indoor conditions of diffused sunlight, a slightly lower plane of malignancy obtained in a group of rabbits kept in constant darkness but that a very low level prevailed in the group exposed to constant light. The particular type of lighting (Mazda lamps and mercury arcs in crown glass) employed in this experiment, however, precluded its use except during the colder months, so that a different arrangement was necessary to permit experiments at other seasons of the year.

In the present paper, 4 experiments are reported in which were

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studied the effect of constant illumination with Cooper Hewitt mercury arcs in crown glass and the effect of constant darkness upon the general course and manifestations of the malignant disease.

Methods and Material.

The experiments were carried out from October 1, 1925, to April 12, 1926. 3 animal rooms with similar environmental conditions other than those of lighting were employed. These rooms which will be referred to as the light, the dark and the control or unaltered room, respectively, have already been described in connection with the 1st experiment (1). It is sufficient in the present report merely to call attention to the lighting arrangement of the light room which was the only feature in the equipment of the 3 rooms that was changed.

A *constant* source of light was furnished by 13 Cooper Hewitt low pressure mercury arcs, Type P, in crown glass arranged in 3 superimposed rows on an iron frame placed in the center of the room. All other light was excluded. The rabbit cages were placed in racks on either side of and parallel with the mercury arcs. The intensity of light reaching the cages was not entirely uniform, those in the central portions of the racks receiving more light than those at the top and bottom or at the ends. The average intensity was 200 foot candles.

The spectrogram of the mercury arcs in crown glass shows that the range of light rays is from 3022 to 5790 Ångström units with the majority falling in the 3650 and 5790 portion of the spectrum.

The temperature of the 3 rooms was satisfactorily maintained at 70° to 75°F. and the humidity of each varied with that of the outside air.

The rabbits employed were males, chosen from a selected stock and were matched as to breed and type. The majority were approximately 8 to 12 months old. Each animal was separately caged and all were fed a similar diet of hay, oats and cabbage.

The experiments were all conducted in the same manner. Comparable groups of rabbits were placed in each room at the same time where they remained for the duration of the experiment. After varying intervals, each group was inoculated with the tumor. All inoculations were made in one testicle with 0.3 cc. of an emulsion of an actively growing primary tumor. This neoplasm is considered to be of epithelial origin (3).

Frequent examinations of the rabbits were made, special attention being given to the general physical condition of the animals, the character of the primary tumor and the detection of metastases recognizable during life. In addition, the rabbits were weighed at regular intervals. Certain animals which developed signs and symptoms of impending death were killed by an injection of air into the marginal ear vein. The experiments were terminated 2 months after inoculation at which time all surviving animals were killed. A complete postmortem examination of each rabbit was made with special reference to the condition of the primary tumor,

the incidence of metastatic tumors and the number, distribution, size and state of these growths.

The observations thus obtained have been used as a basis for evaluating the effect of the particular light condition upon the general level of malignancy. The results are considered from a group standpoint in order to minimize the factor of individual animal variation.

The method used in analyzing the data on metastatic foci obtained at postmortem examination requires some explanation. First, the sites or foci of secondary tumors, not their actual number, are counted and hence the expressions "foci of metastases," "metastatic rate" or "distribution of metastases." On the other hand, the number of metastases detected during life refers to the actual number found.

Second, in order to classify the character of the disease in such general terms as high, moderate and low levels of malignancy, the metastatic foci have been grouped in the following divisions:

I. Suprarenals and eyes................................. 4 possible foci
II. Extensions and implantations to the retroperitoneal and
     mediastinal tissues, omentum, mesentery and parietal
     peritoneum.. 19   "   "
III. Lungs and pleura, liver, kidneys and pancreas........... 5   "   "
IV. Skin and subcutaneous tissue, superficial lymph nodes,
     muscles, heart and pericardium, bones and bone marrow,
     glands of internal secretion with the exception of the su-
     prarenals, the spleen and the central nervous system.... 30   "   "

The reasons for this classification have been stated in the paper describing the results of the first experiment (1). Suffice it to say here that in instances of low malignancy, metastases may be found only in the suprarenals and eyes, while in cases of high malignancy with death in 3 to 5 weeks after inoculation there may be a widespread distribution of tumor to many tissues and organs including the skin, muscles, bones and glands of internal secretion which are practically never involved in instances of low malignancy. An occasional exception to this rule is met with in certain cases of tumors in the cranial sinuses or facial bones which may be the only metastases found.

In the third place, the distribution of metastases has been considered upon a percentage basis of the possible sites of such growths as determined by the actual location of metastases in the first 20 generations of the tumor (4). The percentage involvement for each rabbit was first obtained and these values were then combined on a group basis to form what have been called "summation" values of metastatic foci.

Results.

The results of the 4 experiments consisting of clinical and post-mortem observations are presented in 4 tables and 2 charts, the latter being graphic representations of the distribution of metastatic foci. (Tables I, II, III and IV; Text-figs. 1 and 2.) The experiments are arranged according to the length of exposure to constant illumination or constant darkness prior to inoculation, that is, Nos. I and II, 4 weeks; No. III, 5 weeks and No. IV, 16 weeks. Since the duration of observation subsequent to inoculation was in each case 2 months, the entire period of exposure to these conditions was as follows: Nos. I and II, 12 weeks; No. III, 13 weeks and No. IV, 24 weeks. The results of each experiment are presented in the order of the control, the dark and the light groups, respectively.

Analysis and Discussion of Results.

The purpose of these experiments was to determine whether the constant exposure of rabbits to light supplied by mercury arcs in crown glass and to continuous darkness influenced the general course and character of a malignant tumor. Recognizing the fact that an adequate interpretation of results obtained in experiments of this nature implies a consideration of the tumor process in as comprehensive a way as possible, the present analysis has included all features of the disease which are believed to be significant. In addition, the analysis has been made on a group basis in order to avoid individual animal variation, and the results obtained under the experimental light conditions have been compared, in each instance, with those of appropriate control groups. It will aid to a clearer understanding of the evaluation of these comparisons if a brief description of the disease in the 4 control groups be given first.

General Character of the Disease.—The most striking feature of the tumor process in the control groups was the relatively low level of malignancy which prevailed in all 4 experiments. There was a considerable variation in disease severity among the groups but in none of them was the average plane of malignancy high.

As far as general procedure was concerned, the experiments differed in only 2 particulars, that is, in the time of year at which

they were carried out and second, in the length of exposure to constant illumination and to constant darkness before inoculation which, of course, does not concern the control animals. The dates of inoculation were as follows:

> Experiment I, October   27, 1925.
> "   III, November 2, 1925.
> "   IV, January   29, 1926.
> "   II, February 11, 1926.

The most pronounced disease developed in Experiments I and III but the general plane of malignancy was low as compared with many series observed in previous years. An even less severe disease occurred in the 2nd and 4th experiments, and in the case of No. II, the condition was extremely mild. The generally low level of malignancy as well as the group variations in disease severity are well illustrated by the differences in the mortality rates and in the incidence of metastases determined at postmortem examination as shown by the following data:

	No. of rabbits	Mortality rate	Incidence of metastases
		per cent	*per cent*
Experiment I............................	11	36.4	81.9
" III............................	11	27.3	63.6
" IV............................	5	20.0	40.0
" II............................	10	0	30.0
Weighted average.....................	37	21.6	56.8

Similar variations in metastatic incidence and in the death rate were noted in a study of the first 20 generations of the tumor (5) but there were certain peculiarities of metastatic distribution in the present experiments, which were not wholly characteristic of the disease as previously observed. While this phase of the subject cannot at present be discussed in detail, its occurrence should be noted and its most pronounced feature, namely, metastases to the bones, briefly described because of its bearing upon the experimental results obtained.

As compared with the first 20 generations of the tumor, the incidence and distribution of bone metastases were as follows:

	First 20 generations, tumor	Controls of these experiments
Total No. of rabbits....................	191	37
Incidence of bone metastases...........	15 or 7.9 per cent	6 or 16.2 per cent
Proportion of bone metastases to total foci..............................	13.2 per cent	11.6 per cent
Distribution of bone lesions		
Facial bones........................	12	16
Calvaria...........................	6	0
Thorax............................	4	1
Posterior extremities................	4	2
Spinal column.....................	3	1
Anterior extremities................	1	0
Total bone lesions..................	30	20

Although the disparity in the numbers of animals in the 2 series and the mixed nature of the material comprising the first 20 generations does not strictly permit a comparison, still there are 2 particulars in which the control rabbits of these experiments differed from the larger group. In the first place, there was a much higher incidence of bone lesions—16.2 as contrasted with 7.9 per cent and second, an increase in the proportion of metastases to the facial as compared with other bones (80.0 per cent as contrasted with 40.0 per cent). The ratio of bone metastases to the total number of secondary growths, however, was practically the same in both series. From the standpoint of animal incidence, the bone lesions of the large series were almost equally divided between cases of fulminating malignancy and animals with relatively malignant but slowly progressive tumors. Among the present controls, bone lesions occurred only in cases of comparatively pronounced malignancy. There were no instances in the controls of the 2nd type of malignancy, which is another point in which the character of the disease in these experiments differed from that previously observed. The tumor process of the control rabbits fell, on the whole, into 2 classes, in 1 of which malignancy was fairly well marked while in the other, the disease was very mild as evidenced by the absence of, or the few numbers of, metastases found.

The character of the disease in the groups exposed to constant illumination and to constant darkness did not entirely conform in its general picture to that of the controls, and these differences will be pointed out in the following analysis of the results obtained in these groups.

TABLE I.

Analysis of Clinical Observations.

Experiment No.	Length of exposure before inoculation	Group	No. of rabbits	Primary tumors	Metastases Incidence No.	Metastases Incidence Per cent	Total No.	Mortality No.	Mortality Rate	Mortality Time after inoculation
	wks.								per cent	wks.
I	4	C.	11	All +	5	45.5	13	4	36.4	4, 5½, 6½*, 7½
		D.	12	All +	4	33.3	7	3	25.0	5, 6, 7½
		L.	12	All +	3	25.0	8	1	8.3	3
II	4	C.	10	4 +	0	——	0	0	——	
		D.	9	2 +	2	22.2	2	1	11.1	7
		L.	10	0 +	0	——	0	0	——	
III	5	C.	11	All +	3	27.3	4	3	27.3	4, 5, 5½
		D.	12	All +	2	16.7	4	2	16.6	4, 6
		L.	10	All +	4	40.0	9	3	30.0	4½, 5, 5½
IV	16	C.	5	2 +	2	40.0	10	1	20.0	6½
		D.	5	2 +	2	40.0	4	1	20.0	6½
		L.	4	2 +	0	——	0	0	——	

C. = controls; D. = groups exposed to constant darkness; L. = groups exposed to constant illumination.

* Complicating empyema.

Clinical Observations.—Under this heading are discussed the primary tumor, the development of metastases recognizable during life and the general physical condition of the animals.

A primary tumor developed in all animals of Experiments I and III (Table I). In the others, however, there were a number of instances as shown below in which no definite primary growth occurred.

	Experiment IV	Experiment II
	per cent	*per cent*
Controls..	60.0	60.0
Darks..	60.0	77.8
Lights...	50.0	100.0

Although the intratesticular route of inoculation has yielded an almost uniform incidence of primary tumors, there have been instances in which none developed. An error in technic with the inoculum deposited in the scrotal sac may have been responsible for some of these failures, but in 2 series inoculated in the fall of 1922, the number of negative results were comparable to those of the present experiments (4). In addition, it may be noted that as far as Experiment IV was concerned, the rabbits had been in the laboratory 6 months prior to inoculation and 4 months under the conditions of the experiment—a period probably sufficient for the establishment of a relatively stable state of the organism. It would appear, and this point will later be taken up in greater detail, that the factors of stability or changeability as applied to external conditions are of prime importance in influencing tumor malignancy, the first manifestation of which is the growth capacity of the transplant.

It was also observed in many rabbits kept in the light room that the testicles were reduced in size and became firmer, conditions which would not necessarily be conducive either to initial or to continued tissue growth. Perhaps this factor in connection with the low degree of malignancy prevailing at the time was largely responsible for the completely negative results as regards the primary tumor in the light group of Experiment II.

The non-development of a primary tumor, however, was not necessarily followed by an absence of growths in other parts of the body as shown by the following data compiled from Experiment II:

	Total No. of rabbits	Negative primary results	Incidence of metastases with no primary tumors
Controls...........................	10	6	1 or 16.7 per cent
Darks.............................	9	7	2 " 28.6 " "
Lights.............................	10	10	1 " 10.0 " "

The character of growth of the primary tumor differed under the various light conditions of the experiments. On the whole, the largest tumors developed in the controls, and in general these groups showed a definite tendency towards a longer persistence of active primary growth as compared with an earlier regression in the tumors of the rabbits from the dark and light rooms. The most irregular type of growth occurred in the rabbits kept in the dark room. During the 1st week after inoculation, little difference was noted between the primary tumors of these rabbits and those of the controls, but in the 2nd and 3rd weeks, the resemblance ceased. It then became difficult to determine from clinical observation whether certain tumors in the dark series were growing. Eventually, some tumors grew more rapidly than those in the controls, but in the majority of instances the speed and degree of growth were less marked.

In the groups kept in the light room, on the other hand, the initial development of the primary tumor was definitely slower and less pronounced than in the controls. The majority of the tumors in these groups did not attain the size of those in the controls and in addition, regression took place earlier and was somewhat more rapid. There were certain instances, however, of very actively growing and extensive primary tumors, but they were not as numerous as in either the dark or the control groups.

The condition of the primary tumors at postmortem examination was, on the whole, in harmony with the general character of the disease at the time this was done, irrespective of the group to which the animal belonged. Thus, in rabbits dying within 4 to 6 weeks after inoculation and in which metastases were found to be more or less widely distributed, the primary tumors showed variable amounts of living tissue. On the other hand, the growths in the animals living to the end of the observation period were in the majority of instances either healed or largely necrotic and heavily encapsulated.

The incidence of metastases recognizable during life among control groups varied in the different experiments from 0 to 45.5 per cent (Table I). While there was only 1 control group (Experiment II) in which no secondary tumors were detected, there were 2 such groups from the light room (Experiments II and IV) and the incidence in the others was 25.0 and 40.0 per cent. There was less variation

among the groups kept in the dark room, the values being 16.7 to 40.0 per cent. If the experiments are combined, the incidence of "clinical" metastases was:

	Total No. of rabbits	No. of rabbits with metastases	Rate
			per cent
Controls..................................	37	10	27.07
Darks.....................................	38	10	26.31
Lights....................................	36	7	19.44

The numbers of secondary tumors detected during life were—Controls 27, Darks 17, Lights 17. There was 1 rabbit from the light room (Experiment III) in which a malignant disease developed and the numerous growths in superficial parts of the body of this rabbit considerably augment the total number of clinical metastases of the light groups. On the other hand, there was observed among these rabbits the unusual occurrence of regression of metastases in superficial parts of the body. The tumors of the iris in 2 rabbits healed during the period of observation and in a 3rd animal they were entirely necrotic at postmortem examination.

Continuous exposure to constant illumination of mercury arcs in crown glass for 3 to 6 months did not appear to induce any outspoken deleterious effect upon the general state of health. All rabbits were given a superabundance of food and those in the light room generally ate more than those in the dark or control rooms, particularly during the first weeks of the experiment. The evidence furnished by body weight determinations, however, indicates that conditions of constant darkness were less favorable than those of constant illumination or of variable diffused sunlight. The changes in mean body weights expressed in percentage values of the initial weights are shown in Table II. The first figures for each experiment include all the rabbits in each group while the second values represent only those animals which survived the observation period of 2 months. On the basis of both computations, the gains in body weight of the groups in the light room were approximately the same or exceeded those in the control room while in only 1 experiment, III, was this the case with the rabbits under conditions of darkness, and here the greater gain

of the surviving rabbits was not as much as that of the surviving animals from the light room.   In the other 3 experiments, the groups from the dark room gained much less weight than the controls.

These observations are in general agreement with those obtained in previous experiments in which normal rabbits (6) and rabbits inoculated with *Treponema pallidum* (7) or with the tumor (1) were exposed to conditions of constant light or of constant darkness.

Mortality.—The mortality rates of the control groups ranged from 36.4 per cent in Experiment I to 0 per cent in Experiment II as is seen

TABLE II.

Mean Body Weight.   Percentage Variations.

		Controls	Lights	Darks
		per cent	*per cent*	*per cent*
I	All rabbits..........................	+27.9	+24.5	+11.1
	Survivors..........................	+25.9	+24.1	+22.5
II	All rabbits..........................	+10.6	+12.6	+ 2.2
	Survivors..........................	+10.6	+12.6	+ 6.8
III	All rabbits..........................	+22.4	+24.8	+23.0
	Survivors..........................	+20.9	+34.9	+26.4
IV	All rabbits..........................	+33.5	+39.0	+23.2
	Survivors..........................	+35.4	+39.0	+17.3

in Table I.   The groups from the dark room showed less variations in this respect, that is from 25.0 to 11.1 per cent, and, as will be recalled, this was also the case in regard to the development of metastases in superficial parts of the body.   In 2 experiments, the rates were lower than those of the corresponding control groups (I and III), in another it was the same (IV), while in the last, it was slightly higher (II).

The mortality rates of the light groups also varied considerably, from 30.0 to 0 per cent, but the nature of this variation differed from that of the controls in that there were no deaths in 2 of the groups from the light room while the rates for the other groups were 8.3 and 30.0 per cent as compared with 36.4 and 27.3 per cent for the controls.

The combined mortality rates for the 4 experiments were as follows:

	No. of rabbits	No. of deaths	Mortality rate
			per cent
Controls..................................	37	8	21.6
Darks.....................................	38	7	18.4
Lights....................................	36	4	11.1

From these figures as well as from those of individual experiments, it is seen that the highest death rate occurred among the controls and the lowest in the groups kept under conditions of constant illumination while the rate for the groups from the dark room approximated that of the controls.

The great majority of the rabbits in the 4 experiments survived the observation period of 2 months, but from the distribution and character of metastases found at postmortem examination, it is likely that a number would ultimately have died from the effects of the tumor. In some of these animals, a considerable number of metastatic foci were found while in others, there was comparatively little tumor but this involved such structures as both suprarenal glands or the bones of the jaw or the cranial sinuses and adjacent bones. The number of so called probable deaths was lowest in the controls and highest among the dark groups as shown by the following figures:

	No. of rabbits	No. of actual deaths	No. of probable deaths	Incidence of probable deaths	
				Relative rate	Actual rate
				per cent	*per cent*
Controls......................	37	8	2	5.4	6.9
Darks.........................	38	7	9	23.7	29.0
Lights........................	36	4	4	11.1	12.5

The actual death rate was, as shown above, slightly higher in the control than in the dark room groups, but if the number of probable deaths is also considered, then the highest total mortality occurred in the latter groups. The lowest rates from both standpoints occurred in the groups exposed to constant light.

The character of the disease in the instances of probable deaths will be discussed later in connection with the distribution of metastases, but it may be said now that the outstanding feature of these cases was the high incidence and number of secondary growths in the bones. This is of special interest in connection with the proportion of actual deaths directly caused by bone involvement and here again the dark group showed by far the highest incidence.

	Actual deaths	Fatal metastases to bone		
		No.	Rate	Location
			per cent	
Controls..............................	8	3	37.8	All spine
Darks.................................	7	5	71.4	4 spine
				1 jaw
Lights................................	4	2	50.0	All spine

Postmortem Observations of Metastatic Foci.—Perhaps the most indicative single feature of the tumor process for evaluating the general character of the disease is the distribution and state of secondary growths.

The metastatic foci found at postmortem examination have been analyzed, first, from the point of view of their incidence and total number and, second, from their distribution to certain organs and tissues. As is shown in Table III, the incidence of secondary growths was practically the same in the control and dark groups of 2 experiments, I and II, but it was considerably lower in the case of the light group, while in the 3rd and 4th experiments, it was practically the same in all 3 groups. If the 4 experiments be combined, it is seen from the following figures that there was little difference in the metastatic incidence for the controls and the rabbits from the dark room, but that the rate for the light groups was considerably smaller.

	No. of rabbits	No. with metastases	Incidence
			per cent
Controls.................................	37	21	56.75
Darks....................................	38	23	60.52
Lights...................................	36	14	38.88

The total number of sites involved by secondary growths in the control groups varied greatly, that is, from 6 to 74, and there was much the same variation among the dark groups, that is 13 to 69. In 2 experiments, I and IV, there were slightly fewer foci among the groups from the dark room than among the controls, in No. III, there was the same number, but in the 2nd experiment, there were more in the dark group due to a single instance of a comparatively severe

TABLE III.

Analysis of Metastatic Foci as Observed at Postmortem Examination.

Experiment No.	Group	No. of animals	Incidence		Total No.	Rate		No. of foci in		
			No.	Per cent		Relative	Actual	Deaths	Survivors	
I	C.	11	9	81.9	74	6.7	8.2	26, 19, 14, 6*	4, 2, 1, 1, 1	0 in 2 rabbits.
	D.	12	9	75.0	69	5.8	7.7	19, 6, 2,	24, 6, 5, 5, 1, 1	0 " 3 "
	L.	12	4	33.3	37	3.1	9.3	4	18, 13, 2	0 " 8 "
II	C.	10	3	30.0	6	0.6	2.0	——	3, 2, 1	0 " 7 "
	D.	9	3	33.3	13	1.4	4.3	10	2, 1	0 " 6 "
	L.	10	1	10.0	3	0.3	3.0	——	3	0 " 9 "
III	C.	11	7	63.6	58	5.3	8.3	24, 15, 14	2, 1, 1, 1	0 " 4 "
	D.	12	9	75.0	58	4.8	6.4	12, 6	19, 7, 5, 4, 2; 2, 1	0 " 3 "
	L.	10	7	70.0	44	4.4	6.3	20, 9, 7	3, 3, 1, 1	0 " 3 "
IV	C.	5	2	40.0	34	6.8	17.0	28	6	0 " 3 "
	D.	5	2	40.0	15	3.0	7.5	13	2	0 " 3 "
	L.	4	2	50.0	8	2.0	4.0	——	7, 1	0 " 2 "

* Complicating empyema.

disease in an entire series of very low malignancy. This case was probably a purely chance occurrence which might have been included in either of the other groups, but it is perhaps significant that it did not occur in the light group. The variation in the numbers of metastatic foci was not as marked in the light groups—3 to 44, and there were fewer foci in all light groups than in the corresponding controls. This difference amounted to 50.0 per cent in Experiments I and II, to 24.0 per cent in No. III and to 76.0 per cent in No. IV, in terms of the control values.

The relative rate of metastases, that is the number of foci per rabbit, was uniformly the lowest in the case of all light groups while the rates for the dark groups were somewhat lower than those of the controls except in Experiment II. The value of the relative as contrasted with the actual rate in which only the animals with secondary growths are considered, lies in the fact that the latter is accentuated by the inclusion of 1 or 2 rabbits with numerous metastases. Both rates, however, are useful in furnishing information on the extent of variation in tumor distribution. In these experiments, the control groups were characterized by the most irregular distribution while the groups from the dark room were the most uniform, as shown by the following figures:

Metastatic foci		Controls	Darks	Lights
Relative rate	I........	6.7	5.8	3.1
	II........	0.6	1.4	0.3
	III........	5.3	4.8	4.4
	IV........	6.8	3.0	2.0
Actual rate	I........	8.2	7.7	9.3
	II........	2.0	4.3	3.0
	III........	8.3	6.4	6.3
	IV........	17.0	7.5	4.0

If the numbers of foci in the different experiments be combined, the greatest number together with the highest relative and actual rates is found to have occurred in the controls.

	Total No. of foci	Relative rate	Actual rate
Controls..................................	172	4.65	8.18
Darks.....................................	155	3.89	6.43
Lights....................................	92	2.55	6.56

As compared with the controls, the distribution of secondary growths was somewhat more restricted among the rabbits from the dark room and markedly so in the animals exposed to constant illumination. On the other hand, it would appear from these combined values that as far as the actual rate is concerned, tumor dis-

tribution was as well marked in the light as in the dark series, a fact which alone might suggest comparable degrees of disease severity. That such was not the case has already been shown by such indices as the mortality rate, the incidence of metastases and the total number of metastatic foci, and an additional demonstration is afforded by an analysis of the character of metastatic foci from the standpoint of the organs and tissues involved.

TABLE IV.

Summation Values of Metastatic Foci.

Experiment No.	Group	Suprarenals and eyes	Extensions and implantations	Lungs and pleura, liver, kidneys, pancreas	Skin, subcutaneous tissue, superficial lymph nodes, bones, muscles, endocrine glands, spleen, central nervous system
		per cent	*per cent*	*per cent*	*per cent*
I	C.	250.0	121.2	200.0	103.1
	D.	275.0	136.7	120.0	86.3
	L.	75.0	89.5	120.0	36.7
II	C.	25.0	10.6	60.0	0
	D.	100.0	10.6	60.0	13.2
	L.	0	5.3	40.0	0
III	C.	75.0	105.3	220.0	82.0
	D.	225.0	110.6	140.0	69.8
	L.	275.0	63.3	120.0	26.7
IV	C.	50.0	63.1	80.0	53.3
	D.	125.0	21.1	40.0	13.3
	L.	0	21.1	40.0	6.7

Character of Metastic Involvement.—The method used in determining the relative severity of the disease from the particular organs and tissues involved has already been described in the section on Materials and Methods.

In Text-fig. 1, the summation values of metastatic foci of the dark and light groups are contrasted against those of the controls (Table IV). The most conspicuous feature of this chart are the 4 columns above the base line, representing the greater distribution of metastases to the suprarenals and eyes in 3 dark and 1 light group (Experiments
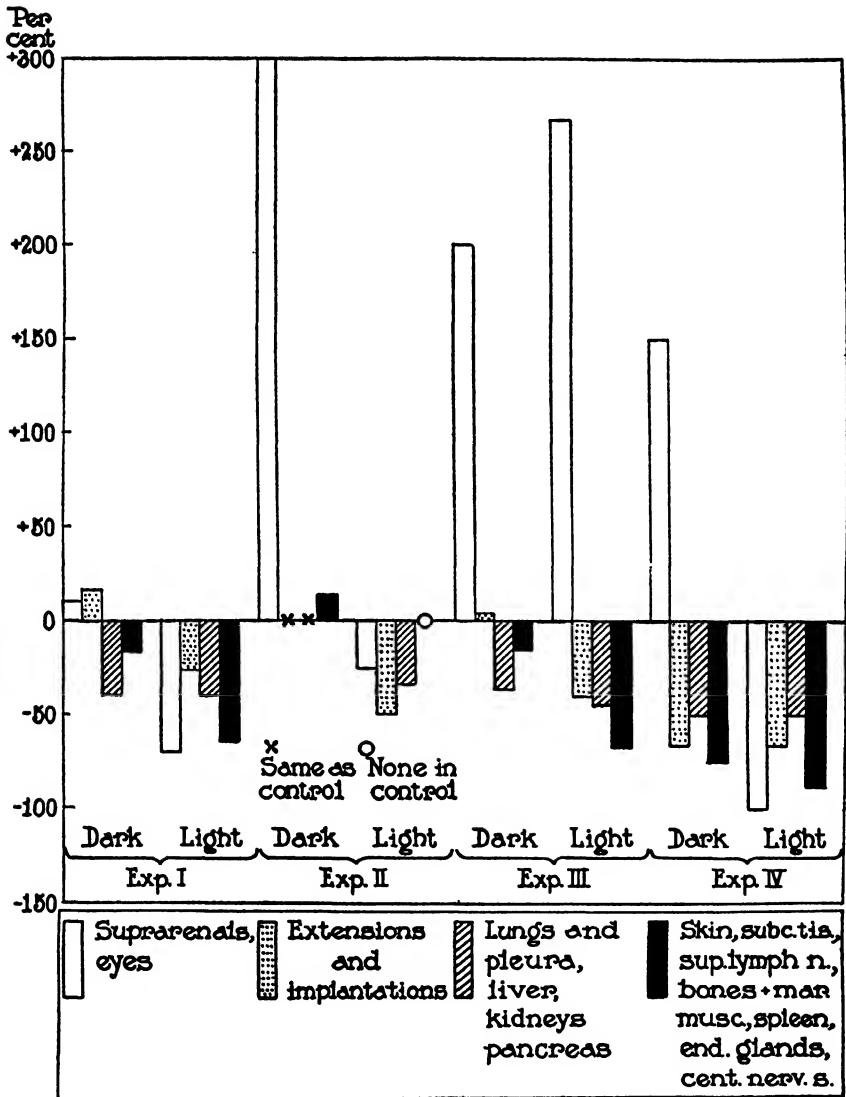
TEXT-FIG. 1.   Summation values of metastatic foci in individual experiments expressed in percentage variations of control figures.

II, III and IV). The disproportionate length of these columns as compared with the others is due, first, to the fact that there are only 4 possible sites for secondary growths in this division as contrasted with 5, 19 and 30 in the others, and, second, to the relative frequency of metastases to these organs in cases of low as well as of high malignancy. In so far as severity of the disease is concerned, however, secondary growths in the suprarenals and eyes are of relative unimportance as compared with those to the viscera, other glands of internal secretion, muscles, bones and skin, which in this chart are represented by the last 2 of each set of 4 columns. In the case of the rabbits exposed to constant light, the distribution of metastases to these significant sites of possible tumor growths was, in each experiment, considerably less marked than it was with the controls as indicated by the length of the columns *below* the base line. A further indication of the comparatively limited distribution of metastases in the light groups is brought out by the position of the first 2 columns representing extensions and implantations to the deep lymph nodes and serous membranes and growths in the suprarenals and eyes. These columns are in all but the one instance mentioned above (Experiment III) *below* the base line.

As far as the groups from the dark room are concerned, reference to the chart shows that in 3 experiments, Nos. I, III and IV, metastases to the viscera, endocrine glands, muscles, bones and skin were not as widely distributed as they were in the control groups, but that the difference was not as marked as it was in the case of the rabbits from the light room. In Experiment II, the distribution of tumor in the divisions of extensions and implantations and of the viscera was the same as in the controls, but was slightly greater in the case of the division of skin, bones, etc. This exception, as has already been mentioned, is due to the occurrence of 1 instance of a comparatively severe disease in a series of low malignancy.

The last chart (Text-fig. 2) in which the summation values for metastatic foci of the 4 experiments have been combined illustrates the outstanding features of the distribution of secondary growths in the rabbits exposed to constant illumination or to constant darkness, as compared with those kept under indoor conditions of variable diffused sunlight. In the case of the animals from the light room,

there was a much more limited distribution of tumor in all 4 divisions of metastatic foci, especially in the last division which is particularly involved in instances of pronounced malignancy. In the case of the rabbits from the dark room, secondary growths were also less widely distributed to 3 divisions of possible tumor foci, but were more frequent in the division of the suprarenals and eyes. As compared with the light groups, the reduction of summation values was not as pronounced.
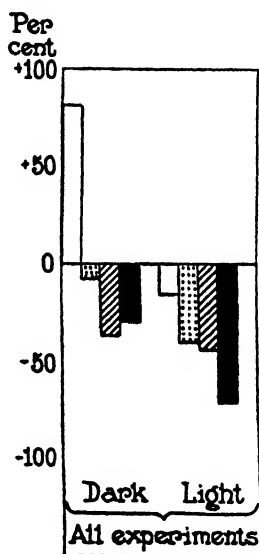
TEXT-FIG. 2. Combined summation values of metastatic foci expressed in percentage variations of control figures.

In discussing the mortality rate, mention was made of the probability that if certain rabbits had been allowed to live beyond the experimental period of 2 months, they would ultimately have died from the effects of tumor growth and statistics were given which showed that the largest number of these cases was found in the groups from the dark room. This aspect of the disease deserves further discussion and may conveniently be considered at this point in connection with the general character of metastatic distribution.

There were 4 instances equally divided between the dark and light

groups of relatively malignant but slowly progressive tumors (Table III). Postmortem examination 2 months after inoculation revealed numerous foci of secondary growth which would certainly have brought about a fatal termination had the animals been allowed to live beyond the experimental period. There were no control rabbits with this type of disease. No difficulty is experienced in recognizing such cases of probable deaths, but there were 11 other rabbits which have been similarly classed because of the location and state of the metastases, although these were comparatively few in number. There were 7 such instances in the dark, 2 in the control and 2 in the light groups, respectively.

The most frequent sites of secondary growths in this type of presumably fatal disease are the suprarenal glands, but in these experiments, there was also an unusual number of metastases to the bones of the face and jaws and the cranial sinuses, particularly in the rabbits from the dark room as shown by the following combined group figures:

	Probable deaths omitting malignant cases	Total foci of metastases	Foci of bone metastases
Darks..........................	7	27	6 or 22.2 per cent
Lights..........................	2	10	1 " 10.0 " "
Controls.......................	2	9	0 " 0 " "

These findings are in line with the greater incidence of bone metastases in the dark groups than in either the control or light series. The observations of individual experiments, as well as the combined values are given below to show that this condition was present in each experiment.

Incidence of Metastases to Bone.

	Controls	Darks	Lights
	No. of rabbits	No. of rabbits	No. of rabbits
Experiment I.........	2 or 18.2 per cent	7 or 58.3 per cent	2 or 16.7 per cent
" II.........	0 ———	1 " 11.1 " "	0 ———
" III.........	3 " 27.3 " "	5 " 41.7 " "	3 " 30.0 " "
" IV.........	1 " 20.0 " "	2 " 40.0 " "	1 " 25.0 " "
Total...............	6 " 16.2 " "	15 " 39.4 " "	6 " 16.7 " "

It will be recalled that in discussing the general character of the disease in the control groups, it was pointed out that the incidence of bone lesions was considerably greater than in the first 20 generations of the tumor. The above data show that in respect to the incidence of this type of metastasis the groups from the light room did not differ from the controls, but that there was a much higher rate among the dark groups. The environment of constant darkness evidently favored the increased tendency toward bone lesions prevailing at this time.

The foregoing analysis of clinical and postmortem data may be briefly summarized at this point before discussing the significance of the results obtained. The tumor process in the control groups exposed to variable diffused sunlight varied from a very mild condition in 1 experiment to a considerably more severe disease in 2 others, but in none of the 4 was a level of well marked malignancy attained. It was much more severe, however, than the disease which developed in the groups exposed to constant illumination and somewhat more so than in the groups kept in constant darkness. As compared with the controls the light groups were characterized by the following features:—an unusual number of failures to obtain a primary growth, less active and less extensive primary tumors, a lower mortality rate, a lower incidence and a smaller number of metastases recognizable during life and at postmortem examination and a much greater restriction in the distribution of these growths. It is significant, moreover, that the influence of constant illumination in the direction of diminished malignancy was observed at times of mild as well as of more severe disease.

A similar comparison of dark groups showed that the growth of the primary tumors was more irregular but on the whole not as pronounced or as persistent as in the controls. On the other hand, the mortality rates and the incidence of metastases found during life and at autopsy were only slightly lower than control values. The number of metastases, however, was smaller, the relative and actual rates were lower and the distribution of these growths was definitely more restricted. An interesting aspect of the tumor process in these rabbits was the tendency toward group uniformity in regard to certain features of the disease. Thus, in the 4 experiments the dark groups as compared

with the control or the light series, showed less variation in the incidence of secondary growths recognizable during life, in the mortality rates and in the relative and actual rates of metastatic distribution. A similar tendency has also been observed in the dark groups of experiments with *Treponema pallidum* (7). Although the influence of constant darkness was exerted toward diminishing tumor malignancy, it was much less pronounced than that of constant illumination, and, furthermore, it had little or no effect in the series in which the disease was of a mild character. It was also modified by the peculiar nature of the tumor process of the 4 experiments, which was characterized by a high incidence of bone metastases despite the moderate or low level of malignancy that prevailed. Conditions of constant darkness were associated with a greatly increased incidence of these lesions which, due to their location in such sites as the spinal column and jaws, were directly responsible for a large proportion of actual as well as of probable deaths.

The results of a previous experiment (1) with constant illumination (Mazda lamps and mercury arcs in crown glass) and constant darkness were of the same order of diminished malignancy but more striking. This was especially true of the group from the light room and while the difference in the case of the dark group was not pronounced, it was definitely greater than in the current experiments. The present analysis would tend to show that the greater severity of the tumor process prevailing at the time of the previous experiment undoubtedly was 1 factor that influenced the particular character of the results obtained. The 2 types of illumination must also be considered, and from this standpoint the combination of Mazda lamps with mercury arcs was more effective than the arcs alone, if one may judge from the single experiment in which the former system was used.

It is obvious that before one can ascribe the effect of diminished malignancy solely to the influence of constant illumination or of constant darkness, other possible factors must be considered. The state or condition of the animal organism prior to exposure to these surroundings is probably of considerable significance and it is conceivable that different states of the host might react in a different manner to these conditions of light and darkness with materially

dissimilar results. Indeed, our explanation of the variations in tumor malignancy is largely based upon the idea that resistant or susceptible states of the host are dependent upon different conditions of animal economy, which may be affected by innumerable factors.

One must also take into account the fact that in these experiments, the animals were subjected to the influence of the factor of change incident to their removal from the variable diffused sunlight of the laboratory to constant conditions of continuous light or of continuous darkness. Little is known of the importance of this factor in relation to the reaction of animals to experimentally induced disease but there are certain observations with this tumor which suggest that the effect of change in external conditions may manifest itself in the direction of increased malignancy. For instance, in 1 experiment dealing with this question, the tumor process in a group of 10 rabbits recently brought to the laboratory was much more severe than in a similar sized group assembled 2 months prior to inoculation.

In order, therefore, that the influence of such a physical force as constant illumination on tumor malignancy be fully effective, it is probable that some sort of initial adjustment or accommodation to the new environment must take place, as experiments begun before its completion would necessarily be complicated by the process whatever it may be. The length of time required for such an adjustment under the conditions of these experiments is not known, but previous work dealing with the changes in organ weights of normal rabbits under similar environmental surroundings, suggested that a 4 weeks' period was associated with relatively stable weights of most organs (6). Consequently, in the majority of tumor experiments, inoculations have been carried out after 4 weeks' exposure to the light or dark conditions. In this connection, the results of an inoculation after 2 weeks' exposure are of interest. The group from the light room showed a less severe disease than the controls, but in the group from the dark room, the tumor process was distinctly more malignant. While the observations of a single experiment are not conclusive, the results of the dark group are in line with what might be expected if the 2 weeks period was not sufficient for the completion of adjustment to this condition. Under these circumstances, the effect of the factor of change would still be operative and if its influence in the

direction of increased malignancy were sufficient, one would expect a more severe disease. On the other hand, a 2 weeks' period was evidently sufficient as far as constant illumination was concerned for the establishment of an adjustment of the animal organism to this condition, or, and this may prove to be the correct explanation, the effect of the light might have been such as to mask or effectually modify the influence of other factors operating in the direction of increased malignancy.

The results of these experiments are interpreted as furnishing evidence in support of the belief referred to in the beginning of this paper, that there is a relation between the factor of light and the manifestations and course of the disease induced by a transplantable malignant neoplasm. The mechanism by which the effects are produced is not known. We have considered the influence of the seasonal factor in this condition and in experimental infections of the rabbit induced by *Treponema pallidum* as operating upon or affecting animal economy and susceptibility or resistance as a functional activity of the animal organism. From this standpoint, it appears that resistance to tumor growth as displayed by rabbits living under certain conditions of constant illumination and to a less extent, in almost constant darkness, was more pronounced than it was in the case of control rabbits exposed to the daily fluctuations of diffused sunlight.

SUMMARY.

Experiments are reported in which an environment of constant and continuous light with a wave-length of from 3022 to 5790 Ångström units, supplied by mercury arcs in crown glass, and of constant darkness, have influenced the course and character of a malignant disease of rabbits induced by a transplantable neoplasm.

Under the influence of constant light the level of malignancy was lower than in control animals living under ordinary conditions of diffused sunlight. Under the influence of constant darkness the level of malignancy was somewhat lower than in control animals but the effect of this environment was modified by the special character of the disease prevailing at this time. The incidence of bone metastases was unusually high, but it was greatly increased in the rabbits kept in constant darkness.

These observations furnish experimental evidence in support of ihe belief that there is a correlation between the external factor of light and the manifestations of an experimental malignant disease.

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ETIOLOGY OF OROYA FEVER.

VIII. Experiments on Cross-Immunity between Oroya Fever and Verruga Peruana.

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A common etiologic origin of Oroya fever and verruga peruana was first indicated experimentally by the production of verrucous lesions in monkeys (*Macacus rhesus*) by inoculation of cultures of *Bartonella bacilliformis* isolated from the blood in a fatal case of Oroya fever.[1] Subsequent experiments with the same strain[2,3] gave evidence that the type of disease induced by the parasite was dependent upon two factors, the virulence of the strain, and the degree of susceptibility of the individual animal, various grades of infection being induced in the experimental animals, from a moderate or extensive local skin affection to a severe systemic disease terminating in fatal anemia. More recently,[4] there has been isolated from the skin lesion of verruga peruana a microorganism morphologically and serologically identical with *Bartonella bacilliformis* and having similar pathogenic properties. It remained to be proven that animals which had recovered from infection with the Oroya strain of *Bartonella bacilliformis* would be able to resist infection with the verruga strain.

The disease induced by *Bartonella bacilliformis* in *M. rhesus* is chronic in nature, but complete recovery may take place and is evident from (1) the subsidence of the febrile reaction, (2) the disappearance of the skin lesions, (3) the sterility of the blood and lymphatic glands, as shown by cultivation experiments, and finally (4) immunity to the homologous strain. All of the ten monkeys

[1] Noguchi, H., and Battistini, T. S., *J. Exp. Med.*, 1926, xliii, 851.

[2] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 697.

[3] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 715.

[4] Noguchi H., *J. Exp. Med.*, 1927, xlv, 175.

(nine *Macacus rhesus* and one chimpanzee) selected for the present investigation had been shown by cultivation of blood and lymphatic gland tissue to be free from organisms, and five of them had been proved immune to the homologous (Oroya) strain by one or more previous immunity tests. The final test of immunity against both strains was made with suspensions of nodular tissue from *M. rhesus* 41 (verruga strain, second passage) and *M. rhesus* 36 (Oroya strain, fifth passage), the Oroya strain being inoculated on the right eyebrow and abdominal skin, intradermally and by scarification, the verruga strain similarly on the left side. Both suspensions induced well developed lesions in a control monkey, the verruga strain being decidedly the more virulent locally, as shown in the protocol of this animal. In the ten animals which had previously passed through a course of infection with the Oroya strain no signs of infection could be induced.

M. rhesus 1 had been inoculated on Nov. 16, 1925, intradermally and intravenously, with the first generation cultures of the blood of Case S. A. 15.[1] *Bartonella bacilliformis* was cultivated from the blood several times during the course of disease, and nodules arose at the site of intradermal inoculation. The lesions had disappeared within 2 months, and on Feb. 22, 1926, cultures of blood and lymphatic gland tissue were negative. Mar. 9, 1926, the animal received intravenously and intradermally a mixture of cultures and a suspension of nodular tissue from *M. rhesus* 18.[2] Blood taken on Mar. 31, and Apr. 13 proved sterile. On Apr. 29 the animal was inoculated again, intradermally and by scarification, with a mixture of cultures with a suspension of nodular tissue from *M. rhesus* 25.[2] On May 19, minute lesions appeared to be developing on the eyebrows, but the slight indurations disappeared, and blood and lymphatic gland tissue taken on June 1 yielded no cultures. The final test with both Oroya and verruga strains was made on June 3, the inoculation being made intradermally and by scarification. No lesions developed.

M. rhesus 2 was first inoculated Dec. 8, 1925, and had passed through a protracted course of infection with the Oroya strain.[1] The blood had yielded cultures on several occasions, and the nodules at the sites of intradermal injection on the eyebrows were still present on Mar. 27. They had disappeared by Apr. 27, and the blood and lymphatic gland tissue taken on that date were negative by culture test. On Apr. 29 the animal was reinoculated with a mixture of cultures with a suspension of nodular tissue from *M. rhesus* 25.[2] No signs of local or systemic infection appeared, and blood and lymphatic gland tissue proved sterile on June 1. On June 3 the animal was inoculated intradermally and by scarification with both the Oroya and the verruga strains. Blood and lymph node cultures made June 22 and July 13 proved sterile. No local lesions developed.

M. rhesus 3 had been inoculated with the same material as *M. rhesus* 2 and had passed through a similar course of disease. Only traces of the local lesions were visible on Mar. 8, 1926. On Mar. 9 the animal was inoculated intradermally and by scarification with a suspension of nodular tissue from *M. rhesus* 18.[2] Blood taken on Mar. 22 yielded cultures of *Bartonella bacilliformis*, and small but definite lesions developed at the sites of local inoculation. These had disappeared by Apr. 28, however, and the blood was sterile. On Apr. 29 a suspension of nodular tissue from *M. rhesus* 25[2] and cultures were introduced intradermally and by scarification. No local reactions resulted, and on May 19 and June 1 blood and lymphatic gland tissue were sterile. On June 3 the animal received the inoculation of both the Oroya and the verruga strains, intradermally and by scarification. The blood remained sterile, and no local lesions developed.

M. rhesus 12[5] had been inoculated on Jan. 19, 1926, intravenously, subcutaneously, and by scarification with cultures derived from Monkeys 4, 6, and 7. The blood was positive by culture on two occasions, and there was high fever for 1 week, but the local lesions were never large, and they disappeared within a month. On Mar. 25 blood and lymphatic gland tissue were sterile. On Apr. 22 the animal was inoculated with a suspension of nodular tissue from *M. rhesus* 18,[2] cultures derived from Monkeys 7 and 20, and citrated blood of *M. rhesus* 25.[2] The blood was negative by culture on May 27, and lymphatic gland tissue was negative on June 1. No lesions developed. On June 3 the final test with both the Oroya and the verruga strains was made. The blood and lymphatic tissue were sterile when tested on June 22, and no lesions developed.

M. rhesus 16 had received two injections (Feb. 10 and Feb. 15, 1926) of killed cultures of *Bartonella bacilliformis*. No reaction resulted. On Mar. 6 active suspensions of the skin lesions from *M. rhesus* 5[2] and *M. rhesus* 18 were injected intradermally and by scarification. By Apr. 5 a large nodule had developed on the abdomen. On Apr. 14 it was larger and deep cherry red. By Apr. 28 the lesion had practically healed. On Apr. 29 the animal received cultures and a suspension of nodular tissue of *M. rhesus* 25[2] intradermally. Small papules appeared at the sites of injection on May 22, but they disappeared, and on June 1 no lesions were apparent. Blood and lymphatic gland tissue taken on this date were sterile. The final test with both the Oroya and the verruga strains was made on June 3. No lesions developed. The blood was sterile when tested on June 21.

M. rhesus 18[2] had been inoculated on Feb. 15, 1926, intradermally and by scarification with a suspension of nodular tissue from *M. rhesus* 5. Extraordinarily extensive lesions had developed by Mar. 17, and blood taken on Mar. 18 yielded cultures of *Bartonella bacilliformis* in dilutions as high as 1:100,000. The lesions persisted for many weeks but had become very small, pale, and fibrous by the middle of June. Blood and lymphatic gland tissue taken on May 15 yielded no cultures. No reaction followed the inoculation of the Oroya and verruga strains on June 3, and on June 22 the blood and lymphatic tissue were sterile by culture test.

M. rhesus 22, inoculated Mar. 5, 1926, intradermally and by scarification on

[5] Noguchi, H., *J. Exp. Med.*, 1926, xliv, 729.

eyebrows and abdomen with nodular tissue of *M. rhesus* 5.   Apr. 26, blood diluted 1:1,000 yielded cultures of *Bartonella bacilliformis*.   A nodule had developed on the right eyebrow, and by May 6 it was very large and pedunculated.   The lesion had disappeared by June 1, and blood and lymphatic gland tissue taken on that date were sterile by culture test.   The final test with the Oroya and verruga strains was made on June 3.   No lesions developed during 4 weeks of observation, and the blood was sterile by culture test on June 22.

M. rhesus 26[5] had been infected by the bites of four ticks previously fed on *M. rhesus* 18, the ticks having been transferred from Monkey 18 to Monkey 26 on Mar. 29, 1926.   Lymphatic gland tissue (inguinal) taken on Apr. 14 yielded cultures of *Bartonella bacilliformis*, as did also blood taken on Apr. 24.   On May 28 blood and lymphatic tissue were both sterile.   The animal was inoculated on June 3 with both the Oroya and the verruga strains.   No lesions developed during 6 weeks of observation.   The blood was negative by culture test on June 22.

M. rhesus 31 was inoculated intradermally Apr. 5, 1926, with a suspension of testicular tissue of Rabbit 1744[6] and nodular tissue of Dog 1.[6]   Blood taken on Apr. 28 yielded cultures in a dilution as high as 1:10,000.   The nodules were well developed at this time.   May 22, the nodules were reduced in size.   May 28, blood culture sterile.   June 1, lesions had disappeared, blood and lymphatic tissue failed to yield cultures.   June 3, final test with Oroya and verruga strains.   No reaction. Blood sterile on June 22.

Chimpanzee (*Pan leucoprymnus*), had been inoculated Jan. 29, 1926, intradermally, subcutaneously, and by scarification, with mixture of cultures and suspension of nodule of *M. rhesus* 3.   Blood positive in dilution of 1:10,000 Feb. 23, 1:100 Mar. 12.   Large nodules developed on eyebrows and abdomen.   The animal had recovered completely by May 1, and neither blood nor lymphatic gland yielded cultures of *Bartonella bacilliformis*.   On June 3 the animal was tested for immunity against the Oroya and verruga strains in the same way as the nine *rhesus* monkeys. No lesions developed, and blood culture was negative on June 28.

The following protocol illustrates the effects produced in a non-immune control by inoculation of the materials used on June 3 for the final test against the Oroya and verruga strains of *Bartonella bacilliformis*.

M. rhesus 45 was inoculated June 3, 1926, with the same materials and in the same manner as the recovered monkeys, the Oroya strain being inoculated on the right eyebrow and abdominal skin, the verruga strain at corresponding sites on the left side.   Several minute nodules were recognizable at the sites of inoculation on

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[6] Both the testicular tissue of the rabbit and the nodule of the dog yielded cultures of *Bartonella bacilliformis;* the report of these and other experiments will be published later.

the left side within 10 days and had attained considerable size (8 × 20 mm.) 32 days after the inoculation. The reactions at the sites of inoculation with the Oroya strain did not become noticeable until 3 weeks after inoculation. The nodules reached their maximum size (4 × 8 mm.) by the middle of July, never attaining the extent of the lesions on the left side. All the lesions were similar in character, however, being of the usual subcutaneous, *mular* type. Cultures made with blood withdrawn on June 28, 1926, 25 days after inoculation, yielded growth of *Bartonella bacilliformis* in a dilution of 1:100,000. All the lesions had healed by the middle of August.

The experiments demonstrate that *rhesus* monkeys which have recovered from infection with the Oroya strain of *Bartonella bacilliformis* are completely immune to the verruga strain. The results warrant the conclusion that the strains from Oroya fever and verruga peruana are identical, and that both conditions are manifestations of infection with *Bartonella bacilliformis*.

The duration of the infection in the present series of animals varied from 2 to 5 months. The cultural test of lymphatic gland tissue appears to be extremely important in determining whether or not the animal is free from infection, as has already been shown in another series of experiments,[7] in which it was found that two animals of a group of four which had apparently recovered still harbored *Bartonella bacilliformis* in certain organs 48 and 58 days, respectively, after inoculation. In this earlier series of animals convalescence was established as early as 30 days in some instances, while in others the infection was still active 68 days after inoculation. The duration of a fatal infection varies from 25 to 57 days.[7]

SUMMARY.

Nine monkeys (*Macacus rhesus*) and a chimpanzee which had recently recovered from an infection with the Oroya strain of *Bartonella bacilliformis* were tested for immunity against the verruga strain of *Bartonella bacilliformis* as well as against the homologous strain. Complete immunity to both strains was demonstrated. The result establishes the identity of the strains and is in agreement with the result of comparative serological study.

[7] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 437.

The criteria of recovery include not only the subsidence of febrile reactions and local lesions but also a negative result of cultural tests of blood and lymphatic gland tissue.

Recovery may occur as early as 1 month after inoculation, but in most instances a period of 2 to 5 months is required for the completion of convalescence.

COMPARISON OF A VIRUS OBTAINED BY KOBAYASHI FROM CASES OF EPIDEMIC ENCEPHALITIS WITH THE VIRUS OF RABIES.

BY E. V. COWDRY.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In 1924 there was a sudden outbreak of a form of epidemic encephalitis in Japan with a reported mortality rate of over 50 per cent which resulted in more than 4,000 deaths. During the epidemic Dr. Kobayashi[1] succeeded, after many attempts, in isolating from a single case of the disease a "virus" capable of indefinite transmission in experimental animals and very courteously sent samples preserved in glycerol to Dr. Flexner for study.

This virus is of peculiar interest. In the first place, because it is doubtful whether previous attempts to transmit the virus of epidemic encephalitis to animals have ever been successful.[2] Secondly, on account of the possibility that the virus of Japanese encephalitis may be of a different kind which, perhaps, may be more easily transmitted than the others, and be thus more readily studied.

The virus was isolated by Kobayashi through the subdural inoculation of rabbits with brain substance from a typical case of epidemic encephalitis diagnosed clinically as well as post mortem. The first symptoms were noted in rabbits on the third passage and subsequently in almost 100 per cent of rabbits inoculated subdurally or corneally. In his opinion, it resembles closely the virus of Koritschoner[3] and the virus isolated from dogs by Hoff and Silberstein.[4] He compared it with the fixed virus of rabies and found many and significant points of similarity as well as the following differences:

1. The encephalitis virus occurred, for the most part, in the brain and was not

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[1] Kobayashi, R., *Japan Med. World*, 1925, v, 145.

[2] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

[3] Koritschoner, R., *Virchows Arch. path. Anat.*, 1925, cclv, 172.

[4] Hoff, H., and Silberstein, F., *Z. ges. exp. Med.*, 1924–25, xliv, 257, 268.

demonstrated in the spinal cord or medulla; whereas that of rabies was found chiefly in the spinal cord.

2. When the encephalitis virus was inoculated on the cornea of 6 dogs, none developed the disease; but when 5 were inoculated subdurally, 4 were affected. With the virus of rabies, on the other hand, 2 out of 3 dogs developed characteristic symptoms after corneal inoculation and all after subdural inoculation.

3. The course of the disease produced by the encephalitis virus was prolonged, and that caused by the virus of rabies, acute.

Evidently it is important to extend the researches of Kobayashi to determine if possible the exact nature of his virus. The following immunological study was therefore made of the encephalitis virus which he sent us and of a fixed rabies virus obtained through the courtesy of Dr. Anna Williams of the New York City Department of Health.

EXPERIMENTS.

In order to test the cross-immunity, if any, existing between the encephalitis virus and the virus of rabies a procedure suggested by Dr. Williams' assistant, Mr. Tyler, was employed with slight modifications.[5]

The viruses were inoculated intracerebrally into a series of young rabbits. The brains of the animals which showed the most characteristic symptoms were used for the preparation of standard viruses of both kinds, which were preserved in ampules of non-alkaline glass. The M.L.D. were then ascertained by the intracerebral inoculation of guinea pigs weighing about 250 gm. These proved to be the same for both viruses, namely $\frac{1}{15,000}$ gm. of brain substance, although the incubation period was uniformly longer in the case of the encephalitis virus, which, on intramuscular injection, was much less potent than the rabies virus. For the first cross-immunity studies two series of 20 guinea pigs of approximately the same weight (250 gm.) were selected.

Series A was given a Pasteur treatment, consisting of 20 daily subcutaneous injections of inactivated fixed rabies virus. Inactivation was effected by making up an 8 per cent suspension of brain substance in 1 per cent carbolic acid in saline solution, and by incubating this at 37°C. for 24 hours. This was diluted with equal parts of sterile saline and, just before use, was diluted again 1 to 3. The amount injected was 0.5 cc. The "vaccine" was tested and found to be free from contaminating bacteria.

Series B received Pasteur treatment in the same way with the inactivated encephalitis virus.

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[5] Help received from Mr. Peter Haselbauer was invaluable.

The animals were left for 1 week after the conclusion of treatment before testing for cross-immunity. The injections were all made intracerebrally and the injected viruses were tested for bacterial contamination and found to be free from it. The symptoms of those succumbing were identical following the action of both viruses. After an incubation period of about 3 days, the temperature rose to about 105°F. on the 4th or 5th day. The animals then showed excessive salivation, tremors, convulsions and paralyses. The tem-

TABLE I.

The Action of Encephalitis Virus upon Animals after Pasteur Treatment with Rabies Vaccine.

Experiment Pasteur treatment with fixed rabies vaccine + encephalitis virus	*Control* Pasteur treatment with fixed rabies vaccine + fixed rabies virus	*Control* Untreated normal animals + encephalitis virus	*Control* Untreated normal animals + fixed rabies virus
2 animals 1 M.L.D. virus	2 animals 1 M.L.D. virus	1 animal 1 M.L.D. virus	1 animal 1 M.L.D. virus
2 animals 2 M.L.D. virus	2 animals 2 M.L.D. virus	1 animal 2 M.L.D. virus	1 animal 2 M.L.D. virus
2 animals 3 M.L.D. virus	2 animals 3 M.L.D. virus	1 animal 3 M.L.D. virus	1 animal 3 M.L.D. virus
No characteristic symptoms or deaths	2 animals receiving 2 M.L.D. developed characteristic symptoms and died	The animal receiving 1 M.L.D. was unaffected, but the other 2 developed characteristic symptoms and died	The animal receiving 1 M.L.D. was unaffected, but the other 2 developed characteristic symptoms and died

perature commenced to fall on the 6th or 7th day and became rapidly subnormal—94° or 95°F.—when the animals died.

An examination of the results which are set forth in Table I shows:

1. That six animals which received Pasteur treatment with the rabies virus resisted subsequent inoculation with the encephalitis virus even to the amount of 3 M.L.D.

2. That the Pasteur treatment with the rabies virus produced a measure of immunity against the rabies virus itself.

3. That untreated normal animals generally succumb to the action of the encephalitis virus showing that the sample of encephalitis

TABLE II.

The Action of Fixed Rabies Virus upon Animals after Pasteur Treatment with Encephalitis Vaccine.

Experiment Pasteur treatment with encephalitis vaccine + fixed rabies virus	Control Pasteur treatment with encephalitis vaccine + encephalitis virus	Control Untreated normal animals + fixed rabies virus	Control Untreated normal animals + encephalitis virus
2 animals 1 M.L.D. virus	2 animals 1 M.L.D. virus	1 animal 1 M.L.D. virus	1 animal 1 M.L.D. virus
2 animals 2 M.L.D. virus	2 animals 2 M.L.D. virus	1 animal 2 M.L.D. virus	1 animal 2 M.L.D. virus
2 animals 3 M.L.D. virus	2 animals 3 M.L.D. virus	1 animal 3 M.L.D. virus	1 animal 3 M.L.D. virus
2 animals 4 M.L.D. virus	2 animals 4 M.L.D. virus	1 animal 4 M.L.D. virus	1 animal 4 M.L.D. virus
One 1 M.L.D. animal died of some cause unknown 11 days after injection	One 2 M.L.D. animal died from peritonitis resulting from intestinal perforation. The others were unaffected	One 1 M.L.D. animal was found dead, no characteristic symptoms having been noted	One 3 M.L.D. animal developed characteristic symptoms and died
One 2 M.L.D. animal developed characteristic symptoms and died			
One 3 M.L.D. animal developed characteristic symptoms and died			
Two 4 M.L.D. animals developed characteristic symptoms and died			

virus has retained its potency. The M.L.D. originally determined is too low because during the experiments, which extended over a period

of more than a month, the animals gained on the average 50 gm. in weight, in consequence of which 1 M.L.D. was not effective.

4. That untreated normal animals likewise succumb to the action of the fixed rabies virus indicating, in the same way, that it likewise has retained its potency. The fact that 1 M.L.D. was not effective is to be explained on the basis above mentioned of the increase in weight of the animals.

TABLE III.

The Action of Fixed Rabies Virus upon Animals 6 Days after Last Immunizing Dose with "A" and "B" Encephalitis Vaccines.

Experiment Pasteur treatment with encephalitis vaccine "A" + rabies virus	Experiment Pasteur treatment with encephalitis vaccine "B" + rabies virus	Control Untreated normal animals + rabies virus
2 animals 1 M.L.D. virus	2 animals 1 M.L.D. virus	1 animal 1 M.L.D. virus
2 animals 2 M.L.D. virus	2 animals 2 M.L.D. virus	1 animal 2 M.L.D. virus
2 animals 3 M.L.D. virus	2 animals 3 M.L.D. virus	1 animal 3 M.L.D. virus
One 3 M.L.D. animal died with typical symptoms*	All recovered but one had slight tremors	The animals receiving 1 and 3 M.L.D. died with typical symptoms.* The animal receiving 2 M.L.D. exhibited tremors, and became ataxic, but recovered

* Examined histologically.

It seems clear, therefore, that the action of the encephalitis virus was inhibited by Pasteur treatment with rabies vaccine so that a one-sided immunity exists.

The observations in Table II were designed to show whether the converse also holds. They are arranged in the same manner, but are less concise, because only 3 out of 8 animals, which received the Pasteur treatment with the encephalitis virus, resisted inoculation with the rabies virus. The controls are similar.

A further attempt was then made, along slightly different lines, to answer the same question as to whether it is possible to protect

animals against the rabies virus by Pasteur treatment with the encephalitis virus.

Instead of making one encephalitis "vaccine," as in the earlier experiments, two were made (designated "A" and "B") each from the brain of a typical case of the disease in a rabbit. The dose of vaccine was increased from 0.5 cc., used in the previous experiments,

TABLE IV.

The Action of Rabies Virus 9 Days after Pasteur Treatment with Encephalitis Vaccines "A" and "B."

Experiment Treated with vaccine "A" + rabies virus	Experiment Treated with vaccine "B" + rabies virus	Control Treated with vaccine "B" + encephalitis virus	Control Normal untreated + rabies virus	Control Normal untreated + encephalitis virus
2 animals received 1 M.L.D. virus	2 animals received 1 M.L.D. virus	1 animal received 1 M.L.D. virus	1 animal received 1 M.L.D. virus	1 animal received 1 M.L.D. virus
2 animals received 2 M.L.D. virus	2 animals received 2 M.L.D. virus	1 animal received 2 M.L.D. virus	1 animal received 2 M.L.D. virus	1 animal received 2 M.L.D. virus
2 animals received 3 M.L.D. virus	2 animals received 3 M.L.D. virus	1 animal received 3 M.L.D. virus	1 animal received 3 M.L.D. virus	1 animal received 3 M.L.D. virus
The 2 M.L.D. animal died with typical symptoms*	No characteristic symptoms: all recovered	No characteristic symptoms: all recovered	All died with typical symptoms*	The 1 M.L.D. animal developed tremors but recovered: the others died with typical symptoms*

* Examined histologically.

to 0.6 cc. The M.L.D. were so calculated as to apply to the animals after the period of immunization when each had gained about 50 gm. in weight. Both vaccines were employed in two sets of experiments and each set of animals thus treated was tested for immunity, respectively, 6 and 9 days after the last immunizing dose.

The results of the first set are detailed in Table III and it is evident that both encephalitis "vaccines" conferred a high degree of

immunity against the action of the rabies virus. The second set of experiments, recorded in Table IV, seems entirely to confirm this result. In other words, there is reason to believe that cross-immunity is demonstrable between the virus isolated by Kobayashi from a case of epidemic encephalitis and the fixed virus of rabies.

Although the immunological properties of the two viruses are thus apparently identical the action of the rabies virus is more rapid and on intramuscular injection more fatal. For example, 10 guinea pigs, which received intramuscularly at the back of the neck 0.5 cc. of a 10 per cent brain emulsion containing rabies virus, all developed characteristic symptoms and died. Whereas 10 other guinea pigs of about the same size which received similar doses of the encephalitis virus all lived. Differences of this kind may presumably be explained on the supposition that the two viruses, though of the same nature, possess different degrees of virulence.

Histological Studies.

The brains of 9 animals (marked with an asterisk in the tables) were examined after fixation in Zenker's fluid and coloration with Giemsa's stain. It was found that the lesions caused by the encephalitis virus and the rabies virus were identical.[6] Similarly 2 young dogs injected intracerebrally with the encephalitis virus developed symptoms indistinguishable from the familiar symptoms of

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[6] Some of the rabbits employed exhibited lesions occasioned by another and wholly different disease, namely, spontaneous encephalitis. These lesions, however, do not constitute a source of error, once their existence is recognized, because they are of slow development and are sometimes associated with a specific and easily recognizable parasite.

In this connection it is desirable to supplement a foot-note in a recent paper on the geographic distribution of spontaneous encephalitis (Cowdry, E. V., *J. Exp. Med.*, 1926, xliii, 730). The note is as follows: "In addition, twenty-four Swedish rabbits, which had been sent to Dr. Flexner by Dr. Kling of Stockholm, were thoroughly examined. Two showed encephalitis and nephritis; one, encephalitis only, and another, only nephritis. No encephalitozoa were observed." It should be added that: "seventeen of these rabbits had been inoculated with brain substance sent by him to us, that the lesions mentioned were restricted to these inoculated animals and that the remaining seven rabbits were employed for control purposes and showed neither symptoms nor lesions."

furious rabies and died.   In order to make sure of the comparison a third dog received an intracerebral injection of the street rabies virus and behaved in precisely the same way.   Large and typical inclusion bodies were observed by a variety of methods in the brains of all 3 dogs.

CONCLUSION.

Since the symptoms produced in experimental animals by the encephalitic virus of Kobayashi and by the virus of rabies are similar, and are accompanied by lesions which are indistinguishable, and since a cross-immunity is demonstrable between the two viruses, the conclusion is advanced that the specimen of so called encephalitis virus isolated by Kobayashi is in reality a specimen of rabic virus.

STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VII. ON THE PARTICULATE NATURE OF BACTERIOPHAGE.

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So called bacteriophage, as ordinarily obtained by filtration of broth cultures of susceptible bacteria after the completion of lysis, represents a heterogeneous mixture containing in addition to the active principle, the unaltered ingredients of the nutritive medium, products of bacterial metabolism, complex materials synthesized by bacteria, and liberated into the medium as the result of lysis of bacteria, etc.

Since at least a part of these substances is present in true solution, and since the active principle in all probability is carried by the particles of colloidal size (1), dialysis and differential ultrafiltration may offer means of eliminating from the filtrate the most highly dispersed and soluble ingredients. This is particularly advisable since we have found that such ingredients of the medium as inorganic salts, for example, may greatly increase the deleterious effect of various reagents which might be used in further purification of the active principle (2, 3). Numerous attempts to dialyze the active filtrates have given conflicting results (4–10). Not only have the findings differed when investigators employed membranes prepared of different material, but even when membranes of similar nature were used. Thus, for instance, Stassano and de Beaufort (11) found that phage passes freely through the membranes prepared from solutions of collodion in acetic acid, though such membranes were impervious to enzymes. Bechhold (6), as well as Villa (12), on the other hand, found that phage was kept back completely, even by such comparatively permeable membranes as those prepared from a 2 per cent solution of collodion in acetic acid. Our own findings show that even the more impervious membranes, up to 7 per cent and over, still allow diffusion of an appreciable fraction of the lytic agent.

However, when lytic filtrates were dialyzed through collodion membranes against fresh distilled water under osmotic pressure, it was found that while in general the rate of diffusion of phage was greater with the more permeable membranes, in no instance was it possible to observe a complete diffusion of the active principle.   In all cases only a fraction of the active principle placed inside the membrane could be recovered in the dialysate, irrespective of the length of time during which dialysis was continued.   So that if the dialysate was collected daily and replaced by fresh distilled water, after a few days the active principle no longer appeared in the outer fluid, while the inner fluid continued to show the presence of the bulk of the original active agent.

The following experiment is selected to illustrate this phenomenon.

Dialysis of Phage through Collodion under Osmotic Pressure.

Membranes of cylindrical shape were prepared from collodion dissolved in the mixture of alcohol and ether (13).   By careful control of the initial amount of collodion used, the size of the surface over which it was spread, the time during which the solvents were allowed to evaporate from the film of collodion prior to its immersion in water, and the rate at which this evaporation of solvents was allowed to proceed, it was possible to prepare membranes of desired permeability. Membranes which were prepared under similar conditions were fairly comparable to one another in their permeability to water.   The latter was determined in terms of time necessary for a given amount of water under constant pressure to pass through a unit of surface of the membrane suspended in air (13).   By this procedure it was possible to check the relative permeability of membranes prepared and to select for duplicate experiments comparable membranes.   Each membrane thus selected was filled with and suspended in water, and sterilized in the autoclave.

One of these membranes, allowing passage of 0.1 cc. of water under a pressure of 10 cm. of mercury over ≳ 44 sq. cm. of surface in 30 seconds, received 30 cc. of the Berkefeld filtrate of a lysed broth culture of *B. coli* (*coli* phage), was mounted on and suspended by means of a rubber stopper (through which was inserted a long glass tube 5 mm. in diameter) in a sterile receptacle containing 2000 cc. of sterile distilled water.   After 24 hours of dialysis, the level of the water in the

Protocol I.

Dialysis of Phage under Osmotic Pressure.

Results of titration of lytic activity in cc.		Duration of dialysis in days										Controls	
		0	1	2	3	4	5	6	8	10	12	15	Original fluid (inside)	Residue after dialysis (inside)
Osmotic pressure (about) in cm..		0	64.5	89	113.5	115.0	118.5	112.5	104.5	94	82	65		
	10^{-10}	−	−	−	−								−	−
	10^{-9}	−	−	−	−								+	+
	10^{-8}	−	−	−	−								+	+
	10^{-7}	−	−		−								+	+
	10^{-6}	−	−											
	10^{-5}		+	−	−									
	10^{-4}		+	+	+	−	−	−	−	−	−	−		
	10^{-3}			+	+	+	+	−	−	−	−	−		
	10^{-2}			+	+	+	+	+	−	−	−	−		
	10^{-1}														
Number of units of phage dialyzed per day		2×10^{5}	2×10^{6}	2×10^{10}	2×10^{9}	2×10^{6}	2×10^{5}	2×10^{4}					$\gtrless 3 \times 10^{10}$	$\gtrless 3 \times 10^{10*}$ $\gtrless 1 \times 10^{10}$†
Total number of units of phage						$\gtrless 2 \times 10^{10}$								

+ = lysis. − = no lysis.

*By titration. †By calculation

glass tube rose to the height of 64½ cm. At this time the fluid in the outer re-
ceptacle was removed and replaced by fresh, sterile, distilled water. This first
fraction of dialysate was titrated for its lytic activity by Appelmans' method, and
the results of the titration were recorded, as shown in Protocol I. After the ex-
piration of a second period of 24 hours, the osmotic pressure was recorded, the
dialysate was again removed and titrated (Protocol I, 2), and the outer container
was again filled with fresh, sterile, distilled water.

A similar procedure was followed at intervals indicated in Protocol I for 15
days, at the end of which time the experiment was terminated and a sample of the
solution inside the collodion bag was titrated, in addition to the sample of the
outer fluid.

It is apparent that over 50 per cent of the active principle was lost
into the outer fluid during the first 5 days of dialysis, but none came
through after this.[1]

These findings suggest at first glance that the particles which carry
the active principle might not be uniform in size, and that the larger
particles were kept back, while the smaller ones came through. How-
ever, since lytic filtrate contains a great deal of colloidal material
(notably proteins), it is also possible that the changes in the rate of
diffusion of phage were caused by a gradual diminution in the perme-
ability of the membrane, due to adsorption of these substances (23).
While regularity of the rate of change in osmotic pressure throughout
the experiment would in itself suggest that there was no abrupt change
in the permeability of the membrane about the 5th day of dialysis,
still this possibility had to be considered before drawing conclusions
as to lack of uniformity in the size of particles carrying active prin-
ciple, particularly as earlier publications lead one to believe that the
active principle consists of particles of approximately uniform size,
approaching 20 $\mu\mu$ in diameter (11, 6, 14, 8).

Possibility of the Partial Occlusion of the Pores of the Membrane.

In order to exclude this possibility, the following experiment was
performed.

30 cc. of a fresh lytic filtrate was dialyzed through one of two collodion mem-
branes, the permeability of which was similar to that of the membrane used in the

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[1] The titration of the activity of the residue does not indicate this loss because
of the tenfold method of dilution in titration.

preceding experiment.   The outer fluid was changed daily and was found to be free from active principle from the 7th day on.   On the 12th day[2] 20 cc. of the residue within the dialyzing bag was removed and placed into a second bag.   At the same time, 20 cc. of fresh lytic filtrate was introduced into the first bag.   Both were suspended in distilled water and dialysis was continued for 4 days, as illustrated in Protocol II.

These results indicate that the residue remaining inside the dialyzing membrane does not diffuse through a fresh membrane of similar

Protocol II.

Permeability of the Membrane after the 6th Day of Dialysis.

Titer of the dialysate in cc.	Dialyzing Bag I 30 cc. of fresh lytic filtrate	
	1st day 1×10^{-4}	
	2nd " 1×10^{-6}	
	3rd " 1×10^{-7}	
	5th " 1×10^{-4}	
	6th " 1×10^{-2}	
	7th " Negative	
	8th " "	
	9th " "	
	10th " "	

On the 12th day 20 cc. of the fluid inside the bag was removed and replaced by 10 cc. of fresh lytic filtrate.   The fluid removed was placed into Bag II.

	Dialyzing Bag I	Dialyzing Bag II
	Residue from dialysis + 10 cc. of fresh lytic filtrate	20 cc. of the residue from Bag I
Titer of the dialysate in cc.	1st day 1×10^{-4}	Negative
	2nd " 1×10^{-4}	"
	3rd " 1×10^{-6}	"
	4th " 1×10^{-2}	"

permeability, and that, on the other hand, the first membrane which held back a portion of the active principle after 5 days of dialysis is permeable to the fresh lytic filtrate.   The inference is that the failure of the active principle to appear in the dialysate after 5 days (Protocol I) is not due to the change in permeability of the membrane, but

[2] 2 days were allowed to elapse in order to obtain the final check on the titration of the dialysate removed on the 10th day.

to the fact that particles carrying the active agent are not uniform in size, and that only smaller particles could pass through the pores of the membrane used in this experiment.

If the above inference is correct—that is, if the fraction of the active principle which dialyzed through is carried by particles of smaller size—then the active principle present in dialysate must pass freely through another membrane of similar permeability. In view of the fact that the dialysate, as obtained in the preceding experiment, is very highly diluted and does not lend itself therefore to such an experiment as the one just outlined, we undertook to fractionate the lytic filtrate by ultrafiltration instead of dialysis as before.
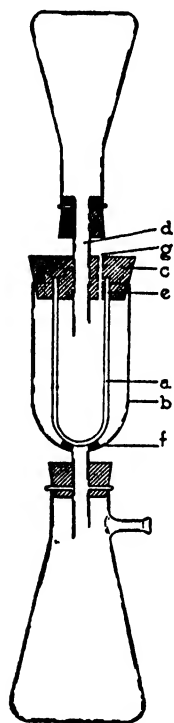
FIG. 1.

The preparation of the membranes and the procedure followed in ultrafiltration are as follows:

Alundum thimbles (Norton RA 360 135 mm. × 45 mm.), glazed to our specifications for a distance of 2 cm. from the rim on the inside surface and for a distance of 2.5 cm. on the outer surface, were used to support a collodion membrane. The latter consists of soluble cotton of any desired concentration, in glacial acetic acid, and is deposited on the inner surface of the alundum under a vacuum. For this purpose a clean (preferably freshly incinerated) thimble (a) is filled with water and adjusted to a Gooch funnel of proper size, (b) by means of a rubber stopper (c) having a small hole in the center (d) and a circular groove fitting the ridge of the thimble (e). The funnel is then placed over a filtration flask connected with the vacuum pump. On the bottom of the funnel rests a thin, perforated, rubber disk (f), protecting the lower end of the thimble against possible injury due to pressure against the funnel (Fig. 1). After all free water is removed by suction, and only traces of water remain adsorbed on the wall of the thimble, a solution of collodion is admitted to the thimble through the hole in the center of the stopper until the thimble is filled with collodion. The suction is maintained for 60 seconds so as to remove all the air from the pores of the thimble and to secure an even membrane without flaws. As soon as connection with the vacuum pump is discontinued, the collodion is quickly poured out of the thimble while the latter is being rotated, to secure an equal thickness of the membrane. During this manipulation care must be taken in handling the thimble not to touch the unglazed surface, to prevent the

production of flaws in the membrane due to uneven evaporation of acetic acid. When no more collodion runs off (2–3 minutes) the thimble is placed under a water faucet and quickly filled with water in order to solidify the collodion. Special precautions must be taken not to wet the outer surface of the thimble before filling it with water, as deposition of water droplets on the outer surface drives the air contained in the clay inward and results in the production of air pockets between the clay wall and the membrane. When the entire thimble is filled with water it may be dropped into a vessel containing running water, for the purpose of washing out the acetic acid. The latter process can be hastened by placing the thimble into the funnel and washing it under negative pressure with warm water.

Protocol III.

Permeability of Collodion to Whole Phage and to Ultrafiltered Fraction Respectively under Negative Pressure.

Titer in cc.	Original phage (control)	Ultra-filtrate A	First washing B	Second washing C	Third washing D	Residue E	Fourth washing F	Ultra-filtrate from A G	Ultra-filtrate from B H
10^{-10}	−					−			
10^{-9}	+					+			
10^{-8}	+					+			
10^{-7}	+		−			+			−
10^{-6}	+	−	+			+			+
10^{-5}	+	+	+			+		−	+
10^{-4}	+	+	+	−		+		+	+
10^{-3}	+	+	+	+		+		+	+
10^{-2}	+	+	+	+	−	+	−	+	+
10^{-1}	+	+	+	+	+	+	+	+	+
10^{-0}	+	+	+	+	+	+	+	+	+

+ = lysis.   − = no lysis.

If the removal of acid is not complete, a portion of the active agent is adsorbed on the membrane during ultrafiltration. Similar loss of the active agent is observed if, instead of adequate removal of acid, the latter is merely neutralized with ammonia (6). It is preferable, therefore, to remove the last traces of acid by adequate washing and electrodialysis (21). Membranes prepared in this way may be used repeatedly, provided they are kept under water when not in use and are sterilized by immersion in alcohol.[3]

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[3] In another connection Dr. Hetler and myself have used this ultrafilter for continuous filtration of large amounts of material by inserting a tube connected with an inverted container through the hole (*d*) in the center of the stopper. In this case a second hole (*g*) which serves to admit the air operates as an automatic regulator for the continuous filling of the thimble from the reservoir above.

Passage of Phage through Collodion under Pressure.

20 cc. of fresh bacteriophage solution was forced, under vacuum of 38 cm., through a membrane of 7 per cent soluble cotton in acetic acid.   The ultrafiltrate thus obtained was collected and titrated to determine the concentration of the lytic agent in it (Protocol III, A).[4]   The residue which failed to pass through the membrane was diluted with 20 cc. of distilled water and subjected to a second filtration through the same membrane.   The ultrafiltrate was again collected and titrated (Protocol III, B), and the residue again diluted with 20 cc. of water and forced through the membrane for the third time.   As will be seen from the protocol (Protocol III, C), the ultrafiltrates obtained at this time, as well as upon a further repetition of the procedure (Protocol III, D), contained only faint traces of active agent.   The residue in the thimble was again diluted to 20 cc. with water, and a sample taken for titration showed that the residue still contained the bulk of the lytic agent (Protocol III, E).   The contents of the thimble were then subjected to filtration through the same membrane for the fifth time, and the ultrafiltrate titrated (Protocol III, F).   At this time the ultrafiltrate collected in the first filtration (Protocol III, A) was added to the residue in the thimble, and subjected to ultrafiltration.   It will be seen (Protocol III, G) that the fluid which came through the membrane at this time contained a comparatively high concentration of active principle.

At the same time the ultrafiltrate obtained from the first washing of the residue (Protocol III, B) was forced through a fresh 7 per cent membrane and the ultra-filtrate thus obtained was titrated.   In this case (Protocol III, H), the ultrafiltrate showed no diminution in activity, as compared with Solution B.

The results of this experiment confirm those obtained earlier, in that they show that only a portion of lytic agent passes through the membrane, that the residue which is held back contains the bulk of the active principle, and yet it can be washed repeatedly without loss of any more active substance into the filtrate.   On the other hand, the filterable portion of the active principle passes freely when added to this residue, or when filtered through a fresh membrane.

These findings suggest that the active principle is carried by the particles, which may differ in size, and therefore that only a part of the active principle which is adsorbed on the smallest particles may pass through a membrane of given porosity.

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[4] Titrations were made both by the method of serial dilution and by the plaque method.   Incidentally it was observed that ultrafiltrates consistently yielded plaques considerably smaller in size than those produced by the agent remaining on the filter.

These experiments were repeated a number of times with the lytic agents active against *B. coli*, *B. dysenteriæ* Shiga, *B. pestis caviæ*, and staphylococcus, and in all cases identical results were obtained. It was found that with all lytic filtrates the proportion of filterable fraction was approximately the same, irrespective of the origin of bacteriophage or bacterial substratum. This observation suggested that nutrient broth itself served as a principal source of the particles capable of passing through the ultrafilter. The validity of this inference seems to be supported by the following experiment, in which the addition of sterile broth to the non-filterable residue of the phage resulted in renewed passage of the active principle through the membrane.

Effect of Broth on the Filterability of Phage.

A portion of lytic filtrate was subjected to ultrafiltration and six repeated washings with water as previously (Protocol III). The ultrafiltrates thus obtained were collected and subsequently titrated for lytic activity (Protocol IV, A).

At the same time another portion of the same phage was subjected to ultrafiltration and three repeated washings with water. At this time the residue in the thimble was diluted to 20 cc. with sterile broth (pH = 7.4) and subjected to ultrafiltration. Following this it was washed with water as before two more times (Protocol IV, B). As a control a third portion of the phage was filtered through a third membrane in exactly the same manner as the second but instead of broth in the fourth washing a buffer mixture of pH = 7.4 was used (Protocol IV, C).

It appears that when sterile broth was added to the residue on the filter (Protocol IV, B), a portion of the phage which was adsorbed on the coarser particles became detached and came through the collodion with the filterable portion of the broth, thus apparently assuming a more dispersed state. This conclusion, however, is valid only if it can be shown that the broth did not modify the membrane itself. This was actually done. When a clean ultrafilter was saturated with broth prior to the filtration of the residue, the latter did pass through to some extent. However, if such an ultrafilter was washed free of broth, it became again impermeable to the phage residue, which would not have been the case if broth had been adsorbed on the membrane in a manner analogous to that of gelatin in the experiments of Hitchcock (23).

Protocol IV.

Effect of Broth on the Rate of Ultrafiltration of Phage.

	10⁻¹⁰	10⁻⁹	10⁻⁸	10⁻⁷	10⁻⁶	10⁻⁵	10⁻⁴	10⁻³	10⁻²	10⁻¹	10⁰
Original phage	−	+	+	+	+	+	+	+	+	+	+
7 per cent membrane A — Ultrafiltrate			−	−	−	+	+	+	+	+	+
First washing with water			−	−	+	+	+	+	+	+	+
Second washing with water					−	−	+	+	+	+	+
Third washing with water							−	−	+	+	+
Fourth washing with water								−	−	+	+
Fifth washing with water									−	−	+
Sixth washing with water									−	−	+
7 per cent membrane B — Ultrafiltrate			−	−	+	+	+	+	+	+	+
First washing with water			−	−	+	+	+	+	+	+	+
Second washing with water				−	−	−	+	+	+	+	+
Third washing with water				−	−	−	−	+	+	+	+
Fourth washing with *broth*	→		−	−	+	+	+	+	+	+	+
Fifth washing with water					−	−	+	+	+	+	+
Sixth washing with water				−	−	−	+	+	+	+	+
7 per cent membrane C — Ultrafiltrate				−	−	+	+	+	+	+	+
First washing with water					−	−	+	+	+	+	+
Second washing with water					−	−	+	+	+	+	+
Third washing with water						−	−	+	+	+	+
Fourth washing with *buffer* (pH = 7.4)							−	−	+	+	+
Fifth washing with water								−	−	+	+
Sixth washing with water									−	−	+

Titer of activity in cc.

DISCUSSION.

Direct (20), as well as indirect (6) microscopic examination of lysed cultures of bacteria, and especially the behavior of lytic filtrates in high dilutions (1) and their ability to cause the appearance of discrete foci of lysis in bacterial cultures on solid media have led to the generally accepted view that the active principle of transmissable lysis (bacteriophage) is present in filtrates of lysed cultures in the form of discrete particles.

By means of ultrafiltration through membranes of known porosity several authors (6, 11, 14) have independently estimated the size of these particles as being approximately 20 $\mu\mu$ in diameter, and consequently having a calculated mass of about $\frac{4}{3} \pi\, 10^{-18}$ gm. (15). Moreover, similar figures were obtained when the size of particles of bacteriophage was estimated by optical methods. Thus, von Angerer (16) found, on the basis of his study of the turbidity of filtrates, that the diameter of particles approximates 20–35 $\mu\mu$. This uniformity of the size of the particles, as found by different investigators and by means of different methods, has in itself appeared to many to be a strong indication in favor of the conception that the particles represent the units of an autonomous organized virus, as originally suggested by d'Hérelle.

However, some experiments, particularly those showing that the active principle is capable of spreading from the focus radially (16), independently of the multiplication of bacteria, and independently of gravity, and that the rate of its spread is conditioned by the density of the medium (1) seem to militate against ready acceptance of such a view.

If the autonomous particulate nature of the active principle is accepted, such spreading must be accounted for either by postulating for the particles the power of locomotion (17), or by assuming that they secrete a diffusible enzyme (18). But independent locomotion, even if claimed to exist (17), (whether it be ameboid or due to the activity of a propelling mechanism, such as flagellum or cilia) would explain only the transport of a given particle in one plane at the time, whereas the active principle spreads from the focus in all directions

simultaneously (16). The secretion of the active enzyme has actually been suggested by d'Hérelle (18), but the evidence presented by him was later found to be inadequate (19).

On the other hand, such radial spreading could be easily accounted for by a working assumption (3) that the observed particulate distribution of phage is only apparent and is due to its ready adsorption on coarser colloidal particles of the medium. That such an assumption is more valid has been suggested by our experiments in which the number of particles endowed with specific activity of phage in a given volume of a filtrate was altered, depending on changes in the degree of dispersion of colloids in the medium (1). Experimental data presented in this paper strengthen this conception further by showing that the particles present in filtrates of lysed cultures of bacteria and endowed with properties of the phage are not uniform in size. Only that portion of the active agent which is carried by the smallest particles was found capable of passing through semipermeable membranes, while the bulk of it failed to pass through even on repeated washing under pressure. While this failure to pass through a membrane is not of itself sufficient evidence to indicate the size of the particles, under the conditions of our experiments, where the effect on the membrane was excluded by proper controls (Protocol III, G and H), failure of the active residue to pass the filter appears to indicate that it is composed of particles too great in size to pass through the largest pores of the ultrafilter employed.

In this connection, it is of interest to note that in our earlier experiments (19), it was found that only a portion of the active agent was carried down with the precipitate produced by the addition of an excess of alcohol at 7°C., and the supernatant fluid usually retained enough active agent to make 1×10^{-5} cc. of it capable of causing lysis of susceptible bacteria. If the rate of precipitation was increased by the addition of electrolytes or by an increase in temperature, the precipitate carried down more of the active principle. It would appear that in this case also only larger particles were precipitated by the alcohol at 7°C., and the supernatant fluid represented that portion of the phage which corresponds almost quantitatively to that fraction which passed the ultrafilter in the experiments reported in this paper.

The fact that active principle can thus be shown to be distributed in the medium in the form of particles of different size does not necessarily deny its autonomous particulate nature, since the phage can conceivably be assumed to be a pleomorphic virus. However, the fact that the addition of broth to the non-filterable residue (presumably composed of units of the agent of larger size) allows it to pass through the membrane which held it back before the addition of broth, is contrary to such an assumption, provided, as we have shown, the permeability of the membrane was not altered by the addition of broth.

Incidentally, in the light of these findings it seems likely that used Berkefeld candles which might have retained some active principle might not give it up into the filtrate if water is passed through them, and yet, if broth is passed instead of water, the filtrates may show some activity due to the detachment of the phage from the coarser particles by the broth. Such an effect of broth would explain the occasional findings of several workers (22) who believe they have caused spontaneous production of phage by filtration of bacterial cultures, heated lytic filtrates, and even sterile broth—substrata assumed by them to be free from bacteriophage before filtration.

SUMMARY AND CONCLUSIONS.

When filtrates of lysed cultures (bacteriophage) are subjected to prolonged dialysis under osmotic pressure against water, the presence of the lytic agent can be detected outside the membrane only during the first few days. The residue remaining inside the membrane contains the bulk of the original lytic agent, and yet it is no longer capable of diffusing into the outer solution.

The interruption of diffusion is shown not to be due to any alteration in the permeability of the membrane. Moreover, the residue fails to diffuse through a fresh membrane of similar permeability, while the dialyzed portion of the phage passes quantitatively through a new membrane. When ultrafiltration under pressure was substituted for dialysis, the residue on the filter could be washed repeatedly with water without giving off into the filtrate any more active agent. However, if broth was substituted for water, a renewed diffusion of the active agent resulted.

These results are interpreted as indicating that the colloidal particles present in the lytic filtrates (and apparently endowed with properties of bacteriophage) do not represent autonomous units of the active agent, but merely serve as a vehicle on which the agent is adsorbed. They vary in size within limits wide enough to permit fractionation by means of ultrafiltration. When the coarser particles retained by the ultrafilter are washed with broth, some of the active agent is detached from its coarse vehicle particles. This agent, now more highly dispersed, is capable of passing the filter which held it back previously.

Preparation of a simple ultrafilter used in these experiments is given in detail.

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STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

VIII. THE MECHANISM OF LYSIS OF DEAD BACTERIA IN THE PRESENCE OF BACTERIOPHAGE.

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With but few exceptions (1, 2), those engaged in the study of bacteriophage agree that one of the essential features of transmissible lysis of bacteria is the fact that the lytic principle undergoes an increase in activity exclusively in the presence of living and actively multiplying bacteria. Without active growth of susceptible bacteria, there is no reproduction of lytic agent and no observed bacterial lysis. Although dead susceptible bacteria readily adsorb the lytic agent, they do not dissolve (3–7). However, Gratia and Rhodes (8) observed that dead staphylococcus may be slowly lysed by the bacteriophage, and that the concentration of the latter in solution probably increases during this process. If live staphylococci are present simultaneously, the lysis of the dead bacteria is more rapid. The lytic effect of live on dead staphylococcus was observed by them also in the absence of bacteriophage[1] and was assumed by them to be due to the utilization of dead bacteria by the live in the process of nutrition (10). The relation of this phenomenon to the one described earlier by these authors (8) was not made clear. A year later Twort (11) independently observed the lysis of dead staphylococcus in the presence of bacteriophage and live bacteria, and suggested that this was made possible through the activation of the bacteriophage by some auxiliary substance contributed by the live bacteria. Because of the importance of these observations on the question of the mechanism of transmissible lysis, we undertook to study it.

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[1] A similar observation has also been made recently by Duran-Reynals (9).

The Lysis of Dead Bacteria.

An 18 hour agar slant culture of *Staphylococcus aureus* "G" was suspended in saline, sealed in an ampoule, and submerged for 1 hour in a water bath at 60° C. to kill the bacteria. 0.3 cc. of this suspension of dead bacteria was placed into each of a series of tubes containing 9 cc. of broth each, and an homologous bacteriophage alone,[2] or together with live staphylococci, was added to some of these tubes, as indicated in Protocol I.

The degree of turbidity resulting, as well as a subsequent increase or decrease in turbidity, was measured by comparison with a specially prepared set of standards in which the turbidity gradually increased from the first to the tenth tube. The figures expressing the results of this and later experiments indicate the serial numbers of the tubes in this arbitrary set of standards to which the turbidity in a given tube was comparable.

Protocol I.

Lysis of Dead Staphylococci.

Tube No.	1	2	3	4	5	6
Broth in cc.	9	9	9	9	9	9
Suspension of dead staphylococci in cc.	0.3	0.3	0.3	0.3	0	0
" " live " " "	0	0	0.1	0.1	0.1	0.1
Phage in cc.	0	0.1	0	0.1	0	0.1
Salt solution	0.7	0.6	0.6	0.5	0.9	0.8
Incubated at 37°C.						
Turbidity of resulting suspensions { Immediately	4	4	5	5	1	1
After 4 hrs.	4	4	8	4	8	0
" 24 "	4	4	10+	1	10+	0

It will be seen from Protocol I that the suspension of dead staphylococci did not undergo lysis by the bacteriophage alone (Tube 2), but when both live staphylococci and the phage were present simultaneously (Tube 4), dead staphylococci underwent almost complete lysis, as shown by the decrease in turbidity of the suspension from that corresponding to Tube 5 of the standard scale to that corresponding to Tube 1—the lowest tube in the scale.

In attempting to repeat this experiment, using other bacteria than staphylococcus (*B. coli* and *B. dysenteriæ*), we failed to obtain clear-

[2] Unless otherwise stated, the bacteriophage used in these experiments was taken out of the stock kept on ice several weeks.

cut results.   This explains the negative results obtained by Doerr and Grüninger (7), who attempted to produce dissolution of dead colon bacillus in the presence of corresponding bacteriophage and of live bacteria.   Moreover, we found that even with staphylococcus, reproducible results can be obtained only if the relative bulk of dead bacteria used is taken into account, as will be shown in the following experiment.

Effect of Variations in Relative Concentration of Phage and of Dead Bacteria.

Two series of eight tubes each received equal amounts of broth (5 cc.).   Each of the tubes of the first series (A) received 0.1 cc. of antistaphylococcus phage diluted in broth 1:1000; and the tubes of the second series (B) each received 0.1 cc. of the same phage diluted 1:100,000.   Following this, the first four tubes of each series received 0.1 cc. each of bacterial suspension containing 80 million of live staphylococci per cc., and gradually decreasing amounts of a suspension of dead staphylococci containing 220 million bacteria per cc.—the first tube of each series receiving 5 cc. of this suspension, the second 0.5 cc., the third 0.05 cc. respectively.   The fourth tube served as control and received no dead bacteria. The fifth, sixth, and seventh tubes of each of the series received respectively 5 cc., 0.5 cc., and 0.05 cc., of the suspension of dead bacteria only and no live bacteria, and the last tube (No. 8) of each series received no bacteria at all.   The volume of liquid in all the tubes was brought to 10.2 cc. with physiological salt solution, and the whole was kept for 24 hours at 37°C.   Changes in turbidity due to lysis of bacteria were recorded, as indicated in Protocol II, and after 24 hours' incubation all the tubes were placed for 30 minutes into a water bath kept at 56°C., and the phage titer of each mixture was determined by the method of Appelmans.

This experiment indicates that both in the series where the initial titer of the phage was 1×10^0cc. (Protocol II, B, Tube 8), and where it was 1×10^{-1} cc. (Protocol II, A, Tube 8), dead bacteria alone adsorbed the entire phage (Tubes 5, 6, and 7 of each series), the rate of adsorption apparently depending on the number of dead bacteria present.   When the concentration of dead bacteria was comparatively low, the adsorption of phage was so slow that when live bacteria which had been added began to multiply, there was sufficient phage left free in solution to produce its usual effect on the live bacteria and to regenerate.   In the case where the initial concentration of phage was lower (Series B), the regeneration of phage took place only in Tube 3, containing the lowest number of dead bacteria.   In Series A,

Protocol II.

Effect of the Number of Dead Bacteria Present in the Mixture on the Regeneration of Phage.

	A								B							
Tube No.	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Broth in cc.	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Phage 1:1000 in cc.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1								
" 1:100,000 in cc.									0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Suspension of live staphylococci in cc.	0.1	0.1	0.1	0.1	5	5	5	5	0.1	0.1	0.1	0.1	5	5	5	5
" " " dead	5	0.5	0.05	0	5	0.5	0.05	0	5	0.5	0.05	0	5	0.5	0.05	0
Salt solution in cc.	0	4.5	5	5	0.1	4.6	5.1	5.1	0	4.5	5	5	0.1	4.6	5.1	5.1

Incubated at 37°C. for 24 hrs. The changes in turbidity during the incubation were recorded at intervals, as indicated below.

	A								B							
Turbidity — Immediately	10	7	5	1	10	7	5	0	10	7	5	1	10	7	5	0
After 4 hrs.	10+	7	4	1–	10	7	5	0	10+	10	5	1–	10	7	5	0
" 24 "	10+	5	2	0	10	7	5	0	10+	10+	3	0	10	7	5	0

All tubes were heated at 56°C. for 30 min. and the titer of phage in each determined.

	A								B							
Phage titer in cc.*	0	10^{-6}	10^{-7}	10^{-7}	0	0	0	10^{-7}	0	0	10^{-7}	10^{-7}	0	0	0	10^9

* The sign 0 = no phage present in 1 cc. of the mixture.
Variation between 10^{-6} cc. and 10^{-7} cc. in the final titer is due to particulate distribution of phage (25).

where the initial concentration of phage was ten times greater, the regeneration of the phage took place both in Tube 3 and in Tube 2. It is evident that the absence of regeneration of phage in Tube 1 of Series A, and in Tubes 1 and 2 of Series B was due to the fact that the phage present in the mixtures was so completely taken up by the dead bacteria that by the time live bacteria had begun to multiply actively (which is essential for the regeneration of phage) after a period of initial lag, there was no free phage left in the mixture. As a result of this, the bacteria in these tubes remained intact, as is evidenced by the fact that the original turbidity of the contents increased slightly, due to multiplication of the live bacteria. On the contrary, in such tubes of each series as exhibited regeneration of phage in the presence of dead bacteria (Tubes 2 and 3 of Series A, and Tube 3 of Series B), the initial turbidity decreased, due to dissolution of dead bacteria.

Specificity of Lysis of Dead Bacteria.

It was seen in the preceding experiments that while phage alone does not cause lysis of dead bacteria, the latter are lysed if phage and live bacteria are present simultaneously and if the concentration of dead bacteria is kept sufficiently low not to interfere with the process of regeneration of the phage. Another condition essential for the lysis of dead bacteria is that the dead and live bacteria be of the same species. This requirement has already been indicated by Twort (11), and we have been able to confirm it. Dead colon bacilli or dead dysentery bacilli were not lysed in the presence of live staphylococcus and staphylococcus phage.

Time Relation of the Lysis of Live and of Dead Bacteria.

The preceding experiments show that live bacteria contribute some specific active principle necessary for the production of lysis of dead bacteria. Twort (11) has suggested that bacteria supply some sort of a complementary substance which activates the phage and disappears as the phage ages. In order to see whether such a substance is present, and to determine more accurately at what stage of lysis of live bacteria it first appears and how long its activity continues, the following experiment was performed.

A series of six tubes containing 10 cc. of broth each received 0.1 cc. of a suspension of live staphylococcus and 0.1 cc. of phage, and were placed in the incubator at 37°. The first tube of the series received at the same time 0.2 cc. of a suspension of heat-killed staphylococcus. At intervals of 2, 4, 6, etc., hours after

Protocol III.

Dissolution of Dead Bacteria Added during and after the Completion of the Lysis of Live Bacteria in the Presence of Bacteriophage.

Tube No.	Test proper						Controls				
	1	2	3	4	5	6	7	8	9	10	11
Broth in cc.	10	10	10	10	10	10	10	10	10	10	10
Suspension of live staphylococci in cc.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0	0
Phage (filtrate) in cc.	0.1	0.1	0.1	0.1	0.1	0.1	0	0.1	0	0.1	0

Incubated at 37°C.

		1	2	3	4	5	6	7	8	9	10	11
Interval before addition of the suspension of dead bacteria in hrs.		0	2	4	6	24	48					
Suspension of dead bacteria in cc.		0.2	0.2	0.2	0.2	0.2	0.2	0	0	0.2	0.2	0.2
Turbidity	Immediately	4						1	1	4	3	3
	2 hrs. later	4	4					.1	1+	4	3	3
	4 " "	5	3	4				5	1	6	3	3
	6 " "	3	2	2	3			10	0	10	3	3
	24 " "	1	2	6	1	3		10+	0	10+	3	3
	30 " "	1+	3	6	1+	2−		10+	1	10+	3	3
	48 " "	6	6	7	7	3*	7*	10+	4	10+	3	3
	72 " "					6*	6*	10+	6	10+	3	3

* Final results in these tubes are somewhat obscured, due to increase in turbidity caused by the multiplication of the resistant bacteria, but the lysis of dead bacteria has taken place even in these tubes, as suggested by the temporary diminution in turbidity in Tubes 5 and 6.

the beginning of the experiment, other tubes of the series each received in turn 0.2 cc. of the same suspension of dead bacteria. The progress of lysis of live, as well as of dead bacteria, was followed and is recorded in Protocol III in terms of turbidity, by comparison with a standard scale, in which the higher numbers indicate greater turbidity. A fall in turbidity below that of the control tube (No. 11), containing dead bacteria alone, indicates the lysis of dead bacteria. It will be

seen that the initial fall in turbidity in all the tubes of the series (Nos. 1 to 6) is followed by a subsequent increase. This is due to the growth of resistants following the initial lysis of the susceptible live bacteria, so that the results in Tubes 5 and 6 are difficult to interpret on the basis of turbidity.

The results show that dead bacteria are dissolved when added to the live bacteria undergoing lysis, any time within 24 hours after the beginning of the experiment, and probably even later, irrespective of the stage at which the lysis of live bacteria may happen to be. Since the lysis of live bacteria under the conditions of the experiment was completed in the first 6 hours (see Tube 8), and since dead bacteria were dissolved when added even much later, it is evident that the actual lysis of live, is not essential for the dissolution of dead bacteria, and that the products of such lysis alone are capable of causing dissolution of dead bacteria.

However, we have observed that filtrates of lysed cultures which should thus contain these products are inactive against dead bacteria (Protocol I). It was suspected, therefore, that during filtration they might have been kept back by the filter, while phage was able to pass freely. If such were the case, then it would appear that the agent responsible for the dissolution of dead bacteria is distinct from the phage proper and can be separated from the latter because of the difference in their respective diffusibility.

In order to determine if such were the case, an attempt was made to interpose a semipermeable membrane between the live and dead bacteria during the lysis of the former to see whether this procedure would prevent the dissolution of the dead bacteria (12).

Separation of Lysed Cultures into Two Fractions by Means of a Semipermeable Membrane.

A series of cylindrical collodion membranes 15 mm. in diameter and 50 mm. long were prepared under carefully controlled conditions. After hardening in water, these membranes were tested for their relative permeability by measuring the time required for 0.1 cc. of water, under pressure of 10 cm. of mercury, to be forced through the membrane suspended in air. It was found that membranes allowing this amount of water to pass through in from 20 to 30 seconds were suitable for the experiment. A number of such selected collodion bags were filled with and suspended in water, and sterilized in the autoclave for 10 minutes, at 20 pounds pressure. While the autoclaving renders the membrane somewhat less permeable

Protocol IV.

Separation of Phage Proper from the Agent Responsible for the Lysis of Dead Bacteria by Dialysis.

	A		B		C		D	
	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside
Broth in cc.	5	150	5	150	5	150	5	150
Phage "	0.5		0.1			0.1		
Live bacteria.					0.1	·	0.1	
Dead "				0.1		0.1	0.1	0.1
Duration of incubation in hrs.	0 / 5	24	0 / 5	24	0 / 5	24	0 / 5	24
Results — Gross lysis (Inside)			0		0	0	Partial lysis	
Gross lysis (Outside)				Lysis		Lysis		
Phage titer in cc. (Inside)	1×10^{-9}	1×10^{-7}			0	1×10^{-8}		
Phage titer in cc. (Outside)	1×10^{-8}	1×10^{-7}			1×10^{-8}	1×10^{-9}		
Bacteria in millions per cc. (Inside)					163	161	267.5	29
Bacteria in millions per cc. (Outside)						165		96*

* These figures represent averages of several counts made on Breed plate or in a counting chamber.

to water, we have found by experience that bags selected and tested as stated above are uniformly permeable to bacteriophage. At the time of the experiment the dializing thimbles were removed from the water in which they had been sterilized, filled with measured amounts of sterile broth, and placed into suitable containers, with aseptic precautions. In each experiment four dializing thimbles were set up as follows:

I. In the first dializing unit a measured amount of antistaphylococcus bacteriophage was added to the broth inside of the dializing thimble, and a small portion of the resulting dilution of phage was immediately taken out for titration by the method of Appelmans. At the same time a small portion of the broth outside of the bag was likewise removed for titration, and the whole unit was placed in the incubator at 37°C. After an interval of 5 and 24 hours respectively, the titration of the fluid inside and outside the thimble was carried out, and it was found that the lytic agent dialyzed freely under these conditions ((Protocol IV, A).

II. In the second dializing unit 5 cc. of broth were placed inside and 150 cc. of broth outside the thimble as before, following which 0.1 cc. of antistaphylococcus bacteriophage was added to the broth inside the thimble, and 0.1 cc. of an 18 hour old culture of susceptible staphylococcus to the broth outside. The whole unit was placed in the incubator and observed at intervals. It was found that live bacteria placed outside the thimble underwent lysis (Protocol IV, B), just as they do when placed in direct contact with phage without the interposition of the membrane.

III. The third dializing unit was set up exactly as the preceding one, except that both live bacteria and the phage were placed outside, and on the inside only 0.1 cc. of a suspension of dead (heat-killed) staphylococcus. As the experiment proceeded in the incubator at 37°C., lysis of live bacteria took place outside the thimble, and the phage dialyzed into the thimble, where it was demonstrated by titration. However, the actual count of dead bacteria placed into the thimble remained unaffected throughout the experiment (Protocol IV, C).

IV. In the fourth dializing unit the phage was placed outside the dializing thimble and allowed to dialyze for 6 hours into the sterile broth inside. At this time 0.1 cc. of a suspension of live staphylococcus was added to the fluid inside the thimble, and a sample was removed for immediate count, which was found to be 1,000,000 bacteria per cc. Immediately following the removal of the sample, 0.1 cc. of a suspension of dead staphylococcus was added, and again a sample was removed. The bacterial count in this sample indicated the presence of 267,500,000 bacteria per cc. Thus, the initial mixture inside the thimble was composed of 1,000,000 live, and 266,500,000 dead bacteria per cc. From then on the bacterial count was repeated at intervals, and it was found that at the end of 5 hours it had fallen to 29,000,000 per cc., and at the end of 24 hours it had risen to 96,000,000 per cc., due to overgrowth of resistant bacteria. As the initial mixture contained 266,500,000 dead bacteria per cc., it is evident that at the end of 5 hours, practically 90 per cent of the dead bacteria had been dissolved.

These tests indicate that the phage responsible for transmissible lysis of live bacteria is easily diffusible, but that the agent liberated during the lysis of live bacteria and causing dissolution of dead bacteria does not pass through the membrane, so that dead bacteria remain unaffected if the active lysis of live bacteria takes place on the other side of the membrane, even though the bacteriophage can be demonstrated in abundance in the dialysate in which dead bacteria are suspended.

Protocol V.

Effect of Adsorption with Live Bacteria on the Phage Titer and Power to Dissolve Dead Bacteria of Lysed Staphylococcus Cultures.

	A						B
	Dissolution of dead staphylococcus						Titer of phage
Original lysed culture in cc.........	0	5					1×10^{-6}
Supernatant fluid after the first adsorption in cc..................			0	5			1×10^{-3}
Supernatant fluid after the second adsorption in cc..................					0	5	1×10^{-1}
Broth in cc.......................	5	0	5	0	5	0	
Suspension of dead bacteria in cc....	0.2	0.2	0.2	0.2	0.2	0.2	

Covered with toluene and incubated at 37°C. .

Turbidity	Immediately..........	3	3	3	3	3	3
	After 6 hrs...........	3	3–	3	3–	3	3–
	" 24 "	3	1	3	2–	3	1

Separation, by Adsorption, of the Phage from the Agent Dissolving Dead Bacteria.

If freshly lysed cultures of staphylococci contain two active agents, as suggested by the experiment just described, it should be possible to separate them from each other also, by removing the phage from the solution through adsorption on live susceptible bacteria, and leaving in the solution only the agent dissolving dead bacteria.

Accordingly, cultures of Staphylococcus "G" were subjected to lysis by the appropriate bacteriophage, at 37°C. At the same time mass cultures of staphylococcus were grown on the surface of agar in Blake bottles. The next day bacteria

collected from the surface of three Blake bottles were washed by centrifugation, and to the solid mass of bacteria at the bottom of the centrifuge tube were added 20 cc. of lysed staphylococcus culture. At this time bacteria were suspended in the fluid above, by vigorous shaking, and the whole mixture was placed on ice for 2 hours, to allow for adsorption of the phage on bacteria. At the completion of a 2 hour period, the bacteria were thrown down by centrifugation at a high speed, for 1 hour. The centrifuge used for this purpose was supplied with a cooling device, so that lysis of bacteria was prevented during the experiment. A portion of the supernatant fluid was removed for examination of its bacteriophage content, as well as of its ability to dissolve dead bacteria, and to the remainder of the fluid was added another lot of bacteria collected from three Blake bottles. The mixture was shaken, placed on ice for 1 hour, and again bacteria were separated by centrifugation. The supernatant fluid was again tested as before. The results of these titrations are recorded in Protocol V.

As the results of this experiment indicate, it is possible to remove most of the bacteriophage proper by adsorption on bacteria, without affecting the power of the lysate to dissolve dead bacteria.

Dissolution of Dead Bacteria by Filtrates of Lysed Cultures in the Absence of Live Bacteria.

The last two experiments show that lysis of dead bacteria depends upon the presence in the freshly lysed cultures, in addition to the phage proper, of another active agent which does not go through the semipermeable membrane of collodion. This agent may conceivably be held back by the porcelain during filtration, and thus the fact that lytic filtrates, as usually prepared, do not cause the dissolution of dead bacteria becomes explainable. However, we found that the retention of this agent by the filter was complete and constant, while Gratia and Rhodes (8), report that the filtrates exhibit a certain amount of activity against old as well as against dead staphylococcus cultures.

Since the failure of lytic filtrates (bacteriophage) to dissolve old or dead bacteria constitutes, in our opinion, a characteristic which assumes fundamental importance in an attempt to understand the mechanism of transmissible lysis, we felt that it was necessary to determine beyond any doubt whether in the experiments of Gratia the active filtrates contained only the phage, or whether, under the conditions of his experiments, a certain amount of the second agent also had passed into the filtrate. Apart from the possibility that the

efficiency of the filters used by Gratia and ourselves might have been different, it seemed likely, that if large amounts of lysed cultures are filtered through a given filter, its efficiency may gradually decrease, and the substance, which at first is held back, may appear in the filtrate after continued filtration, thus possibly explaining the activity of his filtrates (26).

In order to test this possibility, a flask containing 1 liter of broth received a suitable amount of the suspension of the 18 hour agar growth of staphylococcus and of bacteriophage. The resulting mixture was distributed equally into three smaller flasks and incubated at 37°C. for 2, 4, and 6 hours respectively. At the end of 2 hours' incubation, one of the flasks was taken out and immediately subjected to fractional filtration through a new Berkefeld V candle, under pressure of 60 mm. of mercury as follows: At first 50 cc. of liquid were removed from the flask and filtered. The filtrate was collected into a sterile receptacle. Then a second 50 cc. portion of the contents of the flask was filtered through the same candle and the filtrate collected into a second receptacle. Then followed a third fraction of 50 cc. and so on—five fractions in all being employed. At this point the filter candle was discarded and the five fractions of the filtrate were immediately subjected to examination for sterility,[3] phage content, and for their power to cause lysis of dead staphylococcus, as indicated in Protocol VI, Section I A. At the proper intervals the contents of the other two flasks were similarly filtered, each through its own new filter candle, and the fractional filtrates thus obtained were examined, as indicated in Sections I B and I C of Protocol VI.

As another possibility which could explain discrepancies in the results it seemed to us of interest to inquire also into the rate of deterioration suffered by the agent responsible for the lysis of dead bacteria, under the influence of heat and preservation. In order to elicit the rôle of these factors, all the fractional filtrates (immediately after the removal of a small fraction of each for various tests, as shown in Sections I A, B, and C of the protocol) were divided into two portions each.

One portion of each filtrate was subjected to heating in sealed ampoules and submerged under water at 56°C. for 30 minutes. At the end of this time the tubes were removed from the water bath, cooled quickly in cold water, and the contents were tested for phage content and for their ability to cause the lysis of dead staphylococcus, as indicated in Sections II A, B, and C of Protocol VI.

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[3] This control is essential, for if the filtrate contains live bacteria, the subsequent lysis of dead bacteria, if it occurs, cannot be attributed directly to passage of the active agent, but may be due to the lysis of live bacteria.

Dissolution of Dead Bacteria by the Fractional Filtrates of the Culture of Staphylococcus during Progress of Lysis.

Duration of preliminary incubation......	A (2 hrs.)							B (4 hrs.)							C (6 hrs.)						
Amount filtered in cc...........	Con-trol	50	50	50	50	50	50	Con-trol	50	50	50	50	50	50	Con-trol	50	50	50	50	50	50
Filtrate in cc..........	0	1	1	1	1	1	9	0	1	1	1	1	1	9	0	9	1	1	1	1	1
Sterile broth in cc...........	9	9	9	9	9	9	0	9	9	9	9	9	9	0	9	0	9	9	9	9	9
Dead staphylococcus suspension in cc...........	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Incubated at 37°C.

I

	A (2 hrs.)							B (4 hrs.)							C (6 hrs.)						
Phage titer in cc.........	1×10^{-4}							1×10^{-4}							1×10^{-4}						
Turbidity { Immediately..	4	4	4	4	4	0	0	4	4	4	4	4	0	0	4	4	4	4	4	0	0
{ After 24 hrs..	4	4	4	4	4	3	0	4	4	4	4	3	2	0	4	4	4	2	1	1—	0

Portions of each of the fractional filtrates were heated for 30 min. at 56°C. Mixtures were prepared exactly as above with broth and dead bacteria and incubated at 37°C.

II

	A (2 hrs.)							B (4 hrs.)							C (6 hrs.)						
Phage titer in cc.........	1×10^{-4}							1×10^{-4}							1×10^{-4}						
Turbidity { Immediately..	4	4	4	4	4	0	0	4	4	4	4	4	0	0	4	4	4	4	4	0	0
{ After 24 hrs..	4	4	4	4	4	0	0	4	4	4	4	4	0	0	4	4	4	4	4	0	0

Portions of each of the fractional filtrates were kept at room temperature for 8 days. At the end of this time mixtures were made exactly as above with broth and dead bacteria, and incubated at 37°C.

III

	A (2 hrs.)							B (4 hrs.)							C (6 hrs.)						
Phage titer in cc.........	1×10^{-4}							1×10^{-4}							1×10^{-4}						
Turbidity { Immediately..	4	4	4	4	4	0	0	4	4	4	4	4	0	0	4	4	4	4	4	0	0
{ After 24 hrs..	4	4	4	4	4	0	0	4	4	4	4	4	0	0	4	4	4	4	4	0	0

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The remaining portion of fractional filtrates was allowed to stand in the room at ±25°C. for 8 days.   After this period they were subjected to the test outlined in Sections III, A, B, and C of Protocol VI.

This experiment explains the discrepancy.   It appears that when the filter is new and only a small amount of lysed cultures is filtered, only the phage appears in the filtrate, but as more and more of the solution is forced through the same candle, it becomes less selective and allows the passage of the second agent which is capable of causing dissolution of dead bacteria.[4]   This is illustrated in the protocol by a diminution of the turbidity of bacterial suspensions in the tubes containing the filtrate, as compared with the turbidity of the controls. In the case in which the lysis in the original mixture was allowed to proceed only 2 hours before filtration (Protocol VI, Section I A), this agent appeared late—only after the passage of the fourth fraction— but as its concentration in the solution increased with the progress of lysis of live bacteria, it appeared in the filtrate sooner and in greater concentration, so that in the last section of the experiment (C) traces of the agent were already present in the second fractional filtrate, and in the fourth and fifth its concentration was sufficient to destroy almost all dead bacteria, as shown by the decrease of turbidity from 4 to 1.   Comparison of the results in Sections I, II, and III, on the other hand, indicates that the agent lytic for dead bacteria is present only in freshly prepared filtrates of lysed cultures. ˙If these filtrates are allowed to stand or are subjected to heating, this agent undergoes destruction, while the phage is still present in the solution and is unaffected.[5]

[4] Occasionally, on continued fractional filtration, not only the second active agent but also bacteria appeared in the filtrate, as was indicated by the sterility controls.   Such experiments were discarded and repeated, until sterile filtrates were obtained.   These findings are of especial interest in connection with the statements in the literature, in which the appearance of growth in the filtrates of lysed cultures is attributed to the existence of a filterable stage in the life cycle of bacteria (13, 14, 26).

[5] While in the experiment recorded in Protocol VI the agent causing the dissolution of dead bacteria seems to be completely destroyed on standing or on exposure to heat at 56°C. for 30 minutes, this is not always the case.   Repeating these experiments and employing suspensions of dead bacteria of varying density, we found that this destruction is usually very marked but not complete—with

Failure of Filtrates of a Live Resistant Variant, Grown in the Presence
of Bacteriophage, to Induce Lysis of Dead Staphylococcus.

We have shown in the preceding experiments that bacteriophage has no direct lytic action on dead bacteria. Apparently by causing the lysis of susceptible live staphylococcus, it merely sets free a lytic agent preexisting in the bacteria themselves.

If this inference is correct, the incubation of bacteriophage with a live resistant variant instead of with a susceptible staphylococcus should yield a filtrate that will not lyse dead bacteria. Such an experiment was performed with a resistant variant isolated from the susceptible strain of Staphylococcus "G" used previously. It was found that when the experiment was carried out in a manner identical with that shown in Protocol VI, no dissolution of the dead bacteria occurred. On the other hand, dead resistant bacteria were as susceptible to the lytic action of the agent produced by the live susceptible bacteria as were the dead susceptible organisms themselves.

Evidently the failure of the phage to cause the lysis of resistant staphylococcus precluded the liberation of the lytic agent from the latter. However, when susceptible live bacteria were used, the lytic agent was set free and was able to dissolve either the susceptible or resistant dead staphylococci.

In general, the results of the preceding experiment confirm our earlier observations, namely, that the agent responsible for the transmissible lysis of live bacteria is different and independent from that which causes the lysis of dead bacteria. Moreover, the fact that the latter ferment-like agent appears comparatively late during the lysis of live bacteria, that it is thermolabile, and is inactivated on standing,[•] that it is specific in its action, that it does not go through the collodion membrane, that it passes the filters only with difficulty, and causes dissolution of dead bacteria, suggests the possibility that it may be identical with the autolytic endoenzyme which is set free during the lysis of live bacteria.

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very light suspensions of bacteria some residual activity can still be detected, when heavier suspensions of bacteria show no apparent presence of the lytic agent, as will be shown later (Protocol IX).

[•] This inactivation is probably due to its combination with the products of its own activity.

Analogy between the Agent Dissolving Dead Bacteria and the Autolytic Enzyme.

In order to determine whether this ferment-like agent, destroying dead staphylococcus, can be identified with the autolytic endoenzyme of this organism, we repeated some of the experiments described above, using the enzyme obtained from cultures of staphylococcus grown in the absence of phage.

Protocol VII.

Presence of Specific Bacteriolytic Agent in the Young Cultures of Staphylococcus.

	Dead staphylococci		Dead B. coli		Dead B. pestis caviæ		Dead B. dysenteriæ	
Supernatant fluid from staphylococcus culture in cc.	5	0	5	0	5	0	5	0
Broth in cc.	0	5	0	5	0	5	0	5
Suspension of dead bacteria in cc.	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

Covered with toluene and incubated at 37°C.

Turbidity	Immediately...........	5	5	5	5	5	5	5	5
	After 5 hrs.*...........	2	5	5	5	5	5	5	5

* The contents of these tubes were tested for the presence of phage after the completion of the experiment in order to exclude the possibility of lysis having occurred as the result of accidental introduction of bacteriophage.

The culture of staphylococcus was grown in broth for 18 hours, either aerobically or anaerobically, the bacteria were removed by centrifuging for 1 hour at high speed, and 5 cc. of the supernatant fluid were placed in a tube, to which was added a suspension of dead staphylococcus. Another tube containing 5 cc. of sterile broth received a similar amount of the same suspension of dead bacteria. The initial turbidity of the resulting suspensions was recorded by comparison with the standard turbidity scale. Chloroform or toluene was then added to the contents of each tube as a preservative, and the tubes were incubated at 37°C. At the expiration of 5 hours turbidity was again compared with the standard scale and changes recorded, as illustrated in Protocol VII.

In a series of experiments identical with the one just described, suspensions of dead *B. coli*, *B. pestis caviæ* and *B. dysenteriæ* (Shiga) were subjected to the action of the supernatant fluid from the 18 hour growth of staphylococcus, in order to determine whether the action on dead bacteria was specific, and the results were similarly recorded in terms of turbidity of

the suspensions, immediately after the addition of dead bacteria, and after 5 hours of incubation at 37°C. (Protocol VII).

Thus, in the 18 hour culture of staphylococcus grown in broth in the absence of bacteriophage there was present a specific bacteriolytic agent (presumably enzyme) active against dead staphylococcus. In order to identify more closely this bacteriolytic agent with that which was found to be present in cultures of staphylococcus undergoing lysis by the bacteriophage, we repeated the fractional filtration experiment (see Protocol VI), employing the supernatant fluid of a broth culture of staphylococcus instead of the fluid resulting from the progressive lysis of staphylococcus by the bacteriophage (Protocol VIII). The experiment was carried out exactly as before (Protocol VI) and a detailed description of the procedure has therefore been omitted.

As will be seen, this experiment shows that even after 6 hours of growth the cultures of staphylococcus do not contain enough enzyme in solution for it to be demonstrable by the method used. However, after 18 hours of growth there is a measurable amount of the enzyme present. If these findings are compared with corresponding results in the preceding experiment, it will be observed that in the presence of bacteriophage the enzyme-like substance appears earlier and is present in considerably greater concentration (Protocol VI, Section I), since even the filtrate of 2 hours' growth contains the active agent. This difference might have been expected, since in the presence of phage the rate of growth is more rapid, and also because a number of bacteria are undergoing early lysis, thus setting free the enzyme, whereas in the absence of the phage the enzyme appears in solution, coincident with the late autolysis of bacteria. Moreover, in both cases the first fractional filtrates of the cultures presumably containing the enzyme do not show any activity, and it is only on repeated fractional filtration that the activity becomes demonstrable (compare Protocol VI, Section I A with Protocol VIII, D). Similarly, it was found that exposing to heat the filtrates containing the enzyme, or keeping them for days in the laboratory results in a gradual destruction of the active agent in a manner entirely analogous to that observed in the case of the filtrates of lysed cultures of staphylococcus (Protocol VI, Sections II and III). Here again deterioration of the enzyme

Protocol VIII.

Dissolution of Dead Bacteria by the Fractional Filtrates of the Broth Culture of Staphylococcus.

	A (2 hrs.)				B (4 hrs.)				C (6 hrs.)				D (18 hrs.)			
Duration of preliminary incubation..........																
Amount filtered in cc..............	0	50	50	50	0	50	50	50	0	50	50	50	0	50	50	50
Amount of filtrate in cc............	0	9	9	9	0	9	9	9	0	9	9	9	0	9	9	9
Sterile broth in cc..............	9	0	0	0	9	0	0	0	9	0	0	0	9	0	0	0
Dead staphylococcus suspension in cc..	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

Incubated at 37°C. and turbidity determined at intervals.

	A (2 hrs.)				B (4 hrs.)				C (6 hrs.)				D (18 hrs.)			
Immediately.........	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
After 2 hrs........	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" 4 "........	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
" 24 "........	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2—
" 48 "........	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	1—

appeared complete when its activity was tested with heavy suspensions of bacteria, but when lighter suspensions were used, destruction of the enzyme under the conditions of the experiment was found to be incomplete as will be seen in Protocol IX.

So far as we have inquired into the behavior of the unknown agent appearing in cultures of staphylococcus during lysis under the influence of the bacteriophage, and responsible for the dissolution of dead bacteria, it appears in all respects analogous to the autolytic enzyme which can be obtained from older cultures of staphylococcus grown without the phage.

Protocol IX.

The Effect of Density of Bacterial Suspension on the Outcome of the Test of Activity of the Enzyme.

Filtrate of lysed culture kept for 8 days at room temperature in cc.	5			5			5		
Filtrate of 18 hr. old culture of staphylococcus kept for 8 days at room temperature in cc.		5			5			5	
Sterile broth in cc.			5			5			5
Suspension of dead staphylococcus in cc.	0.05	0.05	0.05	0.1	0.1	0.1	0.15	0.15	0.15

Incubated at 37°C.

	Immediately	2	2	2	4	4	4	6	6	6
Turbidity	After 4 hrs.	2	2	2	4	4	4	6	6	6
	" 24 "	1—	2—	2	3	4	4	6	6	6
	" 48 "	1—	1	2	2	3	4	6—	6	6

The Effect of the Lysis of Dead Bacteria on the Titer of the Phage.

Our experiments show, we believe, conclusively that the enzyme-like agent is distinct from phage, and its early appearance in the culture is merely incidental to the lysis of living bacteria and in no way connected with the activity of the bacteriophage itself. However, in view of the fact that Gratia and Rhodes (8) report probable regeneration of phage during the lysis of dead bacteria, we investigated this point.

For this purpose, live staphylococci and a corresponding phage were introduced into a flask containing 200 cc. of sterile broth, and incubated at 37°C. for 4 hours. Up to that time the lysis had taken place only partially, and the titer of phage had reached 1×10^{-6} cc., as illustrated in Protocol X.   The bulk of the contents of the flask, with the exception of 10 cc., was filtered through a sterile candle in order to thoroughly saturate it, and the filtrate was discarded.   The last 10 cc. of the culture were then filtered through the same candle and to this filtrate—presumably containing both the phage and the agent active for dead bacteria—was added a suspension of dead bacteria.   The turbidity of the resulting mixture was estimated, a sample was taken out for the immediate titration of the phage content, and the tube was placed in the incubator.   After 24 hours of incubation the turbidity and phage content were reestimated.

Protocol X.

Effect of Dead Bacteria on the Phage Titer.

Filtrate in cc	10	0	10
Broth in cc	0	10	0
Suspension of dead bacteria in cc	0.2	0.2	0

Incubated at 37°C.

	Turbidity	Phage titer	Turbidity	Phage titer	Phage titer
		cc.			*cc.*
Immediately	4	10^{-6}	4	0	10^{-6}
After 24 hrs	3—	10^{-1}	4	0	10^{-6}

The experiment shows that during the partial lysis of dead bacteria there was no increase in the phage.   On the contrary, the bulk of the phage disappeared from the solution during the incubation, probably having been adsorbed on dead bacteria.

DISCUSSION.

According to the original conception of d'Hérelle (15), the clearing of bacterial suspensions in the presence of bacteriophage is the result of gradual swelling and eventual bursting of bacteria, due to the accumulation within them of multiplying parasites (*Bacteriophagum intestinale*).   Following this bursting, the young parasites are set free in increased numbers to invade other bacteria, and the débris of ruptured bacterial cells is dissolved by the action of lysin secreted by the parasite (16).

Hence, the increase of the concentration of bacteriophage in solution is preceded by the bursting of bacteria, and the destruction of the latter is considered essential for the regeneration of the phage. However, it has been shown repeatedly that the phage titer of a culture shows an increase before the onset of actual lysis of bacteria, and indeed, under certain conditions, it may reach very high concentration without any lysis of susceptible bacteria (17–21). Thus, while lysis of bacteria is the most striking feature of the d'Hérelle phenomenon, it evidently plays no part in the production or regeneration of the bacteriophage, and when it occurs it is secondary to more essential, though obscure changes in bacterial cells which are accompanied by an increase of phage titer. Just what the nature of the process is by which bacteria undergo complete dissolution in the phenomenon of d'Hérelle has not been definitely established. We have shown (22) that there is no valid evidence of the existence of "lysin," as postulated by d'Hérelle, as a secretion of the ultraparasite (16). Moreover, if bacterial débris is dissolved by an enzyme-like lysin secreted by the *Bacteriophagum intestinale*, one would certainly expect that such a lysin would also dissolve dead bacteria, whereas all the experimental data presented thus far indicate that only live and actively growing bacteria are subject to lysis by the phage.

The findings of Twort seemed to us to have offered a means of solving this problem. We have been able to confirm his observations that dead bacteria remain unaffected by the bacteriophage alone, but that they undergo lysis if, in addition to the bacteriophage, homologous live bacteria are present. We have shown in the experiments reported in this paper that bacteriophage itself takes no part in the dissolution of dead bacteria, but acts merely as an incitant for certain changes occurring in live bacteria and leading to their eventual lysis. The dissolution of the dead bacteria takes place at the expense of a lytic enzyme, set free as the result of lysis of the live bacteria.

These findings, taken with our observations on the viscosity of bacterial suspensions in the presence of bacteriophage (23), lead us to infer that the determining factor in the failure of bacteriophage to bring about dissolution of resistant or old bacteria is to be looked for in the failure of these bacteria to swell under the influence of the phage. Apparently the swelling itself (by dilution of intracellular

contents?), or the bursting of live bacteria as the result of the intake of water, is followed by the dissociation of the intracellular enzyme-antienzyme complex (24), with consequent activation of the autolytic enzyme, which attacks not only the débris of ruptured young bacteria, but if present at the same time, also the dead bacteria.

SUMMARY AND CONCLUSIONS.

We have been able to confirm the observations of Twort as well as of Gratia, that dead staphylococcus may undergo lysis if, in addition to a suitable bacteriophage, there is also present live staphylococcus. Moreover, we have endeavored to ascertain the mechanism of this phenomenon and have found that in order to elicit it it is necessary to control the numbers of live and dead bacteria in the mixture. An excess of dead bacteria interferes with lysis by adsorbing the bacteriophage before it has the opportunity to initiate necessary changes in the live bacteria, so that all lysis is prevented. The phenomenon is specific, that is, the lysis of live bacteria is accompanied by lysis of dead bacteria of the same species only. Lysis of dead bacteria occurs best with staphylococcus, an organism which easily undergoes spontaneous autolysis under appropriate conditions. In the case of B. coli or B. dysenteriæ the lysis of the dead bacteria is uncertain. Dead bacteria need not be present in the mixture at the beginning of the experiment; they will be dissolved if added any time before, during, or after the completion of lysis of live bacteria.

If the test is performed so that a suitable semipermeable membrane is interposed between the dead and live bacteria, the dead bacteria are not dissolved, in spite of the lysis of live bacteria on the other side of the membrane. The agent determining the lysis of dead bacteria is not diffusible, while the principle initiating the lysis of live bacteria diffuses freely and is demonstrably present on both sides of the membrane. The complete independence of the agent causing dissolution of dead bacteria from bacteriophage can also be shown by separating the two agents by means of filtration, or by adsorption on bacteria.

The ferment-like substance responsible for the lysis of dead bacteria is different from the bacteriophage. It is not diffusible through collodion, it is easily adsorbed on clay filters, it is heat-labile, and is inactivated on standing.

An agent possessing identical properties was found in cultures of staphylococcus undergoing spontaneous autolysis in the absence of bacteriophage, but in this instance the agent appeared in the filtrates considerably later than it did when phage was present.

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BACTERIUM LEPISEPTICUM INFECTION.

Its Mode of Spread and Control.

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The experiments outlined in this paper were designed to throw some light on the natural mode of spread of *Bact. lepisepticum* infection and to determine a method for its control. The technique employed is based upon the results of a number of previous observations of the spontaneous disease as it occurs in rabbits (1,*a*, 2) and upon measurements of certain properties of host and microbe under limited but rigidly controlled circumstances (1,*b*). It was hoped, therefore, that by eliminating certain variables and submitting certain conceptions to a critical test under natural conditions, it would be possible to ascertain accurately the factors underlying the various phenomena observed.

The test may be divided conveniently into two parts; first, an effort to keep a certain population of rabbits entirely free from *Bact. lepisepticum* infection; second, a study of the behavior of another population of rabbits exposed to the disease in a random and natural manner. To carry out the first part of the experiment, a specially selected group of animals was chosen and placed in a well isolated room, under the care of an experienced attendant; for the second test, the animals were also chosen with care and placed in an isolated room, but were exposed to the risk of infection by a general rabbit caretaker. The details of each test and the results obtained follow.

Spread of Spontaneous Bact. lepisepticum Infection among a Specially Controlled Group of Rabbits.

Materials.—52 rabbits, 3 months old, weighing 800 to 1000 gm. each, were chosen for this experiment. Thirty-six came from a nearby farm which was reported free from cases of clinical snuffles; sixteen were taken from the Rockefeller

Institute breeding room described below. Animals in this breeding room had been entirely free from *Bact. lepisepticum* carriers for 1 year. Hence it was inferred that the young animals employed in this experiment had never been exposed to *Bact. lepisepticum*. The nasal passages of the 52 animals were cultured three times to discover the presence of *Bact. lepisepticum* carriers. None was found.

The rabbits were placed in separate cages measuring about 12 × 12 × 22 inches in dimensions, and distributed uniformly in four stacks of five cages, and one

Ceiling

D 85F-S	C 92F-S	C 29F-S	E 69F-S	E 97 F-S
C 79F	C 49F	C 69F	C 91F	D 44F
E 25F-S	E 44B-S	E 56B-S	C 75F-S	C 71 F-S
E 75B	E 18F	C 68F	C 21 F	E 77 F
D 62 F-S	E 28 F-S	E 58B-S	A 83F-S	C 44F-S

Left

Complete diet   Incomplete diet   81 F
series   series   Complete diet
Floor   series

Window   Door

Floor   C 67 F-S

E 43B-S	E 12 B-S	F 54B-S	E 16B-S	C 95F
E 45F	E 87 F	E 57 B	E 60B	C 46F
E 55B-S	E 63B-S	E 93B-S	E 41 F-S	F 00F-S
E 90F	E 35F	E 65B	E 36B	C 99F
C 38F-S	E 83F-S	C 83F-S	C 39F-S	D 92B-S

Right

Complete diet, Incomplete diet series
series

Ceiling

TEXT-FIG. 1. Plan of experimental room showing arrangement and location of animals. F following rabbit numbers = outside farm source; B = breeding room source; -S = depilation, October, 1925.

stack of six, on two sides of a room about 10 feet wide and 20 feet long. A window was located at one end of the room, a door at the other (Text-fig. 1). The temperature was maintained as nearly as possible at 68°, except during one definite interval described below. Two distinct diets were employed—a "complete" ration, consisting of oats, first quality hay, and fresh cabbage or carrot, plus 2 or 3 cc. of cod liver oil daily, and an "incomplete" diet of the oats and hay, plus water without any fresh vegetable or cod liver oil. The animals were cared for by attendants who were also in charge of several hundred other rabbits nearby, many of which suffered from snuffles and carried *Bact. lepisepticum*.

Method.—The group of 52 animals was then submitted to the chances of random infection through the vehicle of the caretakers, the bedding, food, insects, etc. Throughout a period of 2 years as many factors as possible were controlled and analyzed. In order to do this with so small a group, every precaution was taken to keep the experimental variables balanced. Thus, the animals from the breeding room were scattered uniformly among those from the farm. The diet groups were likewise evenly distributed so as to include both series of animals and both sides of the room (Text-fig. 1). The population consisted therefore of a group of animals among which was operating a definite number of controlled and balanced variables.

The animals were kept under strict observation. Notes were made of their general health, the presence or absence of snuffles, and other pathological processes. Nasal cultures were taken at frequent intervals (1, *c*) to determine *Bact. lepisepticum* carriers. All animals dying during this period were autopsied carefully.

After the experiment had been in progress for about 10 months, it was decided to modify atmospheric conditions in the room by arbitrarily altering the temperature and humidity. This was done by turning on the steam heat to full strength in the morning, shutting the doors and windows, and turning on the hot water. The room became filled rapidly with water vapor and reached a temperature of about 90°. At night the heat and hot water were shut off and the window opened wide. This caused the temperature of the room to drop to that prevailing out of doors. These extreme measures were employed from October 22, 1925, until February 4, 1926. At the same time the backs of the animals on the top, middle, and bottom rows on each side of the room were depilated from neck to buttocks by means of sodium sulfide (Text-fig. 1).

On December 1 the surviving animals were given intranasally a 16 hour blood broth culture of the virulent Rivers D strain of *Bact. lepisepticum* (1, *d*). And on December 18 to 23 the entire group was killed and autopsied. Cultures were taken from nasal passages, ears, and lungs.

Results.—The results of these observations are shown in Tables I and III and Text-fig. 2. They will be considered in detail with reference to (*a*) the total population, (*b*) the groups on either side of the room, (*c*) the groups from the farm and breeding room, (*d*) the diet groups, (*e*) seasonal factors, including temperature changes and exposure, and (*f*) individual differences in response on the part of the animals.

(*a*) *Total Population.*—The outstanding events referable to the total population are taken from Table I and Text-fig. 2, and charted in Text-fig. 3. *Bact. lepisepticum* carriers appeared in January, 1925, increased at a more or less uniform rate from 10 per cent to 22 per cent in October. *B. bronchisepticus* carriers rose from 4 per cent in March to 31 per cent in May, and fell to a level of about 25 per cent from

TABLE I.

Summary of Findings on Experimental Group of 52 Rabbits.

Side	Diet	Condition	Dec., 1924	Jan., 1925	Feb.	Mar.	Apr.	May	June	Aug.	Oct.	Nov.	Dec.	Mar., 1926	Apr.	May	July	Sept.	Oct.
Left (26)	Complete (16)	Deaths	0	0	0	0	0	0	0	0	0	0	0	1‡	0	0	0	0	0
		Snuffles	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Carriers Bact. lep.	0	1	1	0	3	1	2	1	2	2	—	5	7	9	7	3	7
		Carriers B. bronch.	0	1	1	1	1	7	7	6	5	4	—	6	6	6	9	6	4
	Incomplete (10)	Deaths	0	0	0	0	0	0	0	0	0	0	0	1‡	0	1‡	0	0	0
		Snuffles	0	0	0	1	0	0	0	0	0	1	2	0	0	1	0	0	0
		Carriers Bact. lep.	0	3	6	3	1	2	4	1	3	4	—	6	4	6	3	2	2
		Carriers B. bronch.	0	0	1	1	5	6	4	4	4	3	—	5	3	2	7	5	5
Right (26)	Complete (10)	Deaths	0	0	0	0	0	0	0	0	0	0	1‡	0	0	0	0	1*	0
		Snuffles	0	0	0	0	0	0	0	0	0	1	2	3	3	2	2	3	3
		Carriers Bact. lep.	0	1	0	1	1	1	1	1	2	3	—	6	7	5	5	4	6
		Carriers B. bronch.	1	0	0	0	0	2	2	0	0	1	—	1	1	2	4	3	2
	Incomplete (16)	Deaths	0	0	0	0	0	0	0	1*	0	0	2‡	0	0	0	0	0	0
		Snuffles	0	0	0	0	0	0	0	0	0	0	1	1	1	1	3	1	1
		Carriers Bact. lep.	0	1	1	1	2	3	3	5	4	5	—	9	10	7	10	8	9
		Carriers B. bronch.	0	0	0	0	1	0	1	3	5	1	—	4	3	0	2	5	3

* Otitis media: *Bact. lepisepticum.* † Septicemia: *B. coli.* ‡ Pneumonia: septicemia: *Bact. lepisepticum.*
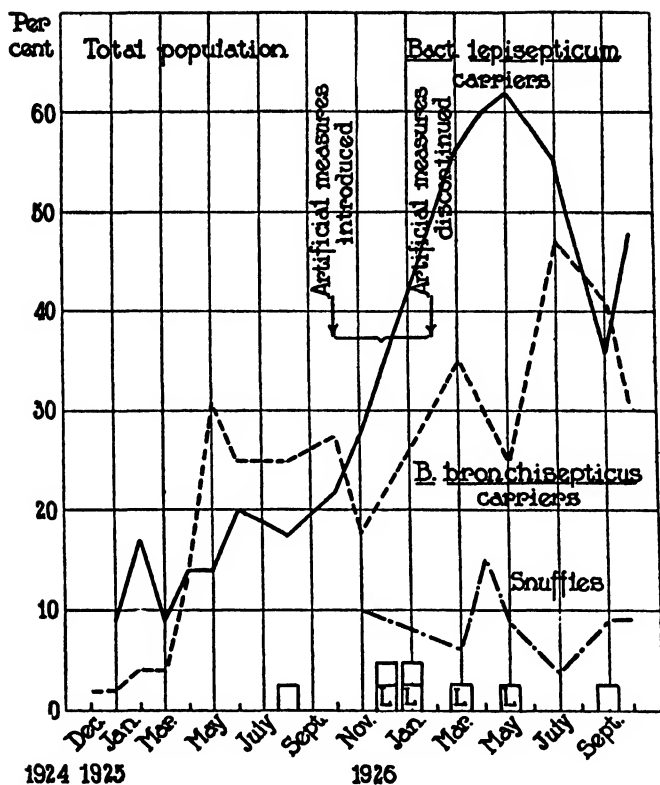
TEXT-FIG. 3. Reaction of the total population to *Bact. lepisepticum* infection.
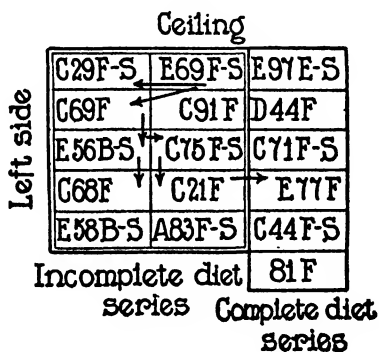
TEXT-FIG. 4. Cage to cage spread of *Bact. lepisepticum* infection from a single focus, January 30, 1925, to June 25, 1925.

June to October. One death occurred in August. This animal showed bilateral otitis media.

During this period and throughout the 2 years of observation, a spot map was kept, showing the location and spread of *Bact. lepisepti-cum* and *B. bronchisepticus* carriers. The early months showed random and scattered foci of *Bact. lepisepticum* infection with a tendency toward local spread. One such focus is charted in Text-fig. 4. The diagram includes the "incomplete" diet series on the left side of
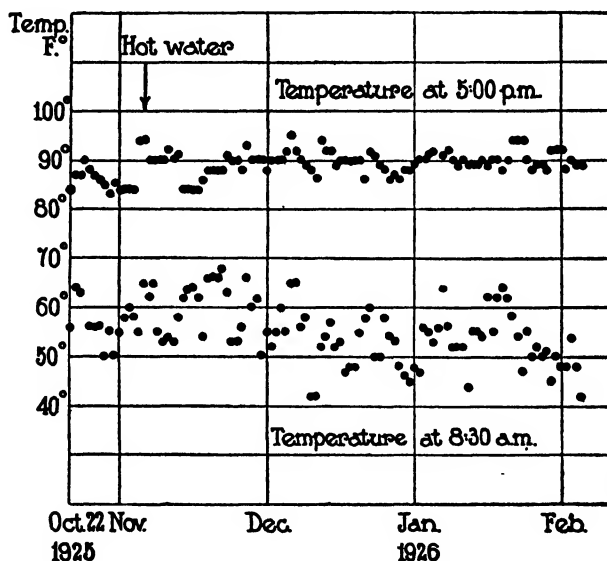
TEXT-FIG. 5. Daily temperature fluctuations in experimental rabbit room.

the room, and six adjacent animals of the "complete" diet series (see Text-fig. 1). The date of appearance of *Bact. lepisepticum* among these animals, recorded in Text-fig. 2, is shown here chronologically by means of arrows.

Apparently the infection started with Rabbit E 69 F-S, and extended to the two adjacent cages, C 29 F-S and C 69 F. From this latter it progressed to E 56 B-S and from there to the three contiguous animals, C 75 F-S, C 68 F, and C 21 F. Finally, it appeared in E 77 F. Evidently, therefore, direct extension of the infection did occur. This possibility appeared the more probable when it was learned that the

attendant always fed the animals in vertical rows, from ceiling to floor. It must be remembered, however, that new foci were appearing throughout the population, and that the attendant was continually passing from the animals in this room to other badly infected stock nearby. Hence, two sources of infection were continually in operation; one, a local spread from established foci in the population, and second, an importation of organisms from without by the attendant.

On October 22, 1925, the artificial temperature fluctuations described above were begun. These procedures continued until February 4, 1926. Daily temperature fluctuations are indicated in Text-fig. 5, in which the 8.30 o'clock morning readings and 5.00 o'clock afternoon readings are recorded. Twice during every 24 hours, therefore, a fluctuation of approximately 35° occurred. Furthermore, on November 6, the hair was removed from the backs of one-half of the entire population (see Text-fig. 1).

These procedures were followed by a striking increase in the percentage of *Bact. lepisepticum* carriers, the appearance of acute and chronic snuffles, and the death of six animals within 7 months. *Bact. lepisepticum* carriers rose to 56 per cent in March, 60 per cent in April, and 62 per cent in May; snuffles appeared and became chronic among about 10 per cent of the population; two animals died in December, two in January, and one in March and May, respectively. Four of the fatalities were due to *Bact. lepisepticum* septicemia or pneumonia. No definite increase of *B. bronchisepticus* carriers was noted during this time. This marked increase in the spread of *Bact. lepisepticum* infection, accompanied by clinical snuffles and a definite mortality, was associated so conspicuously with cold weather and the experimental temperature fluctuations that a causal relation was inferred.

From May to September there was a sharp decline in the percentage of *Bact. lepisepticum* carriers, a decline in the number of cases of clinical snuffles, and in mortality. However, the number of *B. bronchisepticus* carriers increased.

(b) *Left and Right Sides of the Room.*—When the prevalence of *Bact. lepisepticum* infection on the right and left sides of the room is compared (Table I), the same general phenomena are noted. For the first 6 months carriers seemed more abundant on the left side, but during the winter of 1926 the rise was uniform. During the summer

of 1926, however, the carrier rate fell on the left side more rapidly than on the right. Chronic snuffles was more prevalent on the right side, and deaths were about equally distributed.

(c) *Breeding Room and Farm Groups.*—The animals from the breeding room appeared to be somewhat more susceptible than those from the farm (Table II). A summary was made at the end of the experiment of the number of deaths, the number of cases of chronic snuffles, of chronic and occasional carriers of *Bact. lepisepticum*, and of animals free of the infection. 25 per cent of the breeding room animals died, as compared with 14 per cent of the farm animals; 31 per cent of the former showed chronic snuffles, 14 per cent of the latter. About the same relative numbers of each group were chronic carriers, but none

TABLE II.

Comparison of Breeding Room and Farm Rabbits and Depilated and Non-Depilated Rabbits.

Group	Died		Chronic snuffles		Chronic carriers		Intermittent carriers		Free of *Bact. lepisepticum*	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Breeding room (16)......	4	25	5	31	5	31	2	12	0	—
Farm (36)..............	5	14	5	14	9	25	12	33	5	14
Non-depilated (22)......	3	13.5	3	13.5	6	27	10	45.5	0	0
Depilated (28)..........	5	18	7	25	7	25	4	14	5	18

of the breeding room animals was free of infection during the entire period; while 14 per cent from the farm never showed *Bact. lepisepticum.*

(d) *Diet.*—A comparison of the two diet groups is shown in Table I and Text-figs. 6 and 7. Text-fig. 6 shows, with one exception, that throughout the entire period of observation the percentage of *Bact. lepisepticum* carriers among the "incomplete" diet series was higher than that of the group receiving the "complete" ration. However, there was no striking difference in the percentage of chronic snuffles cases, nor in the mortality rates. Therefore, it is difficult to interpret the effect of omitting fresh vegetables from the rabbits' diet for a period of 2 years, further than to state that no deleterious effect on general health could be observed. However, the fact that these

animals were more prone to carry *Bact. lepisepticum* in their nasal passages is of interest from an epidemiological point of view. Differences in host susceptibility, too slight to be recognized clinically, may apparently be determined by the more delicate bacteriological test.
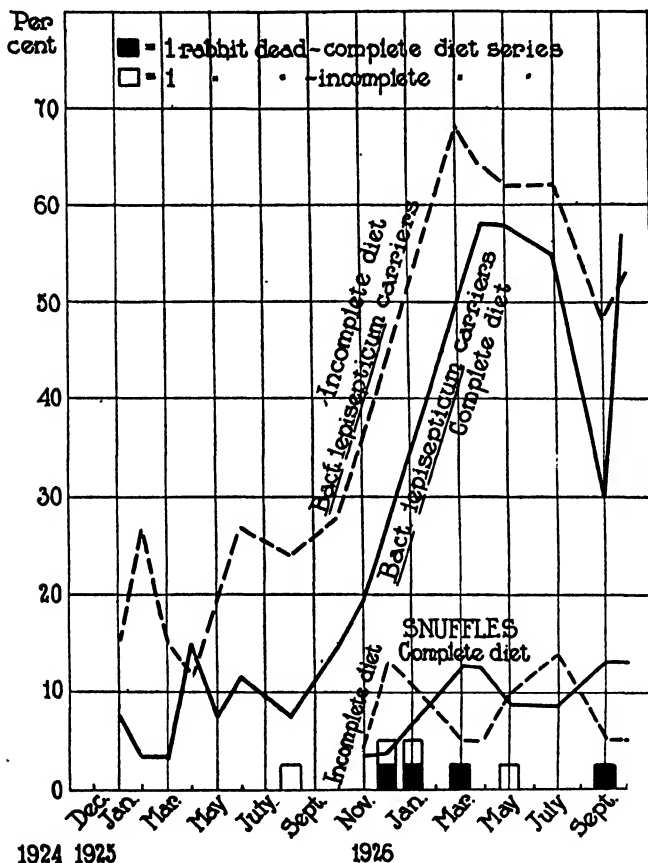

TEXT-FIG. 6. Spread of *Bact. lepisepticum* infection among the "complete" and "incomplete" diet groups.

And this modification of diet, by rendering the individual susceptible to mild infection, whereby he becomes a chronic carrier, may indeed be one influence which, by decreasing host resistance, raises dosage of bacteria available to the population to a dangerously high level.

Text-fig. 7 compares the percentages of *bronchisepticus* carriers in

the "complete" and "incomplete" diet series. No significant differences are apparent.

(e) *Season.*—From the figures available in this experiment, it is difficult to determine any effect of seasonal factors. During the summer of 1925 the percentage of *Bact. lepisepticum* carriers remained moderately low, during the winter of 1926, it rose to a great height,
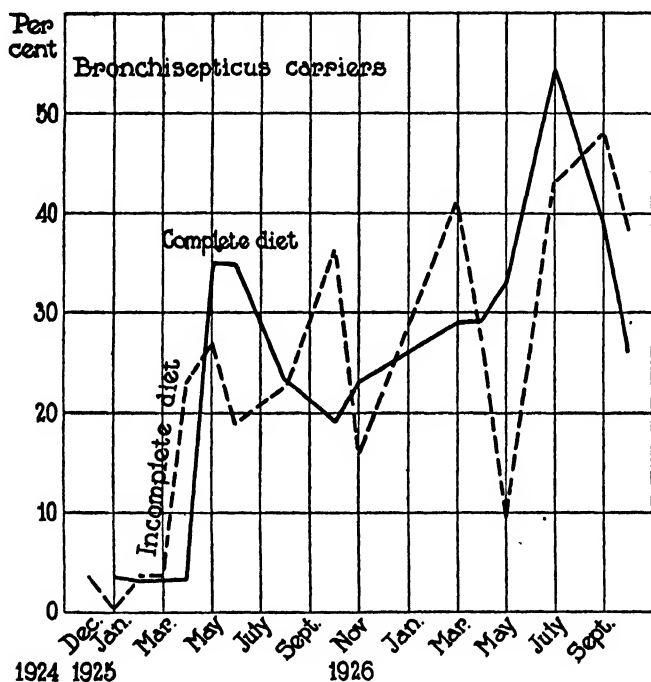
TEXT-FIG. 7. Frequency of *B. bronchisepticus* carriers among the "complete" and "incomplete" diet groups.

and during the following summer declined. However, the complicating factors of artificial temperature changes and depilation make analysis of environmental influences impracticable.

(f) *Depilation.*—The same may be said of the artificial measures of depilation. This was carried out at about the same time that the temperature fluctuations were carried out and was followed by a sharp rise in carrier rate and appearance of chronic snuffles and definite

mortality from *Bact. lepisepticum* septicemia and pneumonia.[1] Probably the three factors, namely, exposure, temperature fluctuation, and seasonal influences were together responsible for the great increase in the prevalence and severity of the disease.

(g) *Behavior of Individual Rabbits.*—Throughout the 2 year period of observation certain animals appeared to be more resistant than others to *Bact. lepisepticum* infection. Hence, at the end of the experiment a final test was employed to emphasize these differences. For this purpose the Rivers D strain of *Bact. lepisepticum* was instilled into the nares of each rabbit. The reaction was slight; two or three acute cases of clinical snuffles developed, but in general the organisms tended to disappear rapidly from the nasal passages (1, e). Evidently continual exposure to infection over a period of 2 years had exerted a selective action in weeding out the less resistant individuals, thus leaving a group relatively resistant and showing little difference in response to this organism.

At the end of the experiment the 52 animals were grouped arbitrarily, according to their behavior during the 2 year period (Table III). Those animals that died composed the first group; those that reacted locally with chronic *Bact. lepisepticum* snuffles, the second; those that became chronic carriers of *Bact. lepisepticum*, the third; intermittent carriers, fourth; and finally, those that failed at any time to show *Bact. lepisepticum* in their nasal passages, fifth. Nine animals were placed in the first group. Four of these died from *Bact. lepisepticum* infection and showed in their lungs and hearts' blood the specific organisms. Ten showed symptoms of chronic snuffles and revealed at autopsy an extensive erosion of the nasal turbinates, with accumulations of pus in the sinuses. Nine of them showed pus in one or both middle ears. The mucoid form of *Bact. lepisepticum* was cultured from these lesions. Besides, from the nasal passages of six, ten to twenty colonies of the experimentally introduced Rivers D strain were found. Fourteen animals were chronic carriers. At autopsy two showed pus in the nasal passages and seven unilateral or bilateral otitis media. The mucoid form of *Bact. lepisepticum* was cultured from all of the ear

[1] Four of the six animals dying between December, 1925, and May, 1926, were depilated; on the other hand, a comparison of the reactions of the two groups as a whole (Table II) shows no notable differences.

TABLE

Summary of Fate of 52

No.	Fate		Clinical condition	Autopsy findings		
				Nasal passages	Ears	Lungs
E 54 B	Died	8/15/25	Torticollis	Normal	Pus, bilateral	Normal
E 83 F–S	"	12/ 7/25	"	"	Normal	Congestion, hemorrhage
E 16 B–S	"	12/12/25	Diarrhea; emaciated	Pus	"	Pleuro-pneumonia
D 85 F–S	"	1/ 8/26	Pregnant	"	"	Congestion
E 36 B	"	1/13/26	Snuffles; emaciated	"	"	Congestion consolidation
E 56 B–S	"	3/ 8/26	Snuffles; diarrhea	"	"	Hemorrhagic consolidation
C 69 F	"	5/21/26	Good	No pus	"	Hemorrhagic
E 90 F	"	9/16/26	Emaciation; torticollis	" "	·Pus, bilateral	Normal
C 37 F–S	"	11/25/26	Cellulitis	" "	No pus	"
E 55 B–S	Killed	12/18/26	Emaciated; snuffles	Erosion; pus	Pus bilateral,	"
E 43 B–S	"	"	Good	" "	" "	"
C 38 F–S	"	"	"	Normal	Normal	"
E 57 B	"	"	"	Erosion; pus	Pus, bilateral	"
E 93 B–S	"	"	"	" "	" left	"
C 30 F–S	"	"	"	" "	" bilateral	"
C 95 F	"	"	"	" "	" left	"
A 85 F–S	"	"	"	" "	" right	"
E 63 B–S	"	"	"	" "	" bilateral	"
E 87 F	"	"	"	" "	" "	"
81 F	"	12/20/26	"	Pus	" "	"
E 60 B	"	"	"	"	Normal	"
E 18 F	"	"	"	Normal	Pus bilateral	"
C 68 F	"	"	"	"	" "	"
E 58 B–S	"	"	"	"	" right	"
E 41 F–S	"	"	"	"	" left	"
D 92 B–S	"	"	"	"	" "	"
C 83 F–S	"	"	"	"	" "	"
F 00 F–S	"	"	"	"	Normal	"
E 44 B–S	"	"	"	"	"	"
E 12 B–S	"	"	"	"	"	"

III.

Experimental Rabbits.

Bacteriological findings				Group
Heart's blood	Nasal passages	Ears	Lungs	
Sterile	No *Bact. lep.*	*B. coli*	Sterile	Deaths
B. *coli*	" " "	Sterile	B. *coli*	"
Bact. lep. M	*Bact. lep.* M	"	*Bact. lep.* M	"
B. coli	" " "	"	B. *coli*	"
Sterile	B. *bronch. Bact. lep.* M	"	*Bact. lep.* M	"
Bact. lep. M	*Bact. lep.* M	"	" " "	"
" " "	B. *bronch. Bact. lep.* M	"	" " "	"
Sterile	No *Bact. lep.* M	—	—	"
"	B. *bronch.*	—	—	"
—	*Bact. lep.* D ++ M Inf.	*Bact. lep.* M	—	Chronic snuffles
—	" " " ++ " "	" " "	—	" "
—	" " " ++	—	—	" "
—	B. *bronch.* Inf. *Bact. lep.* M Inf.	*Bact. lep.* M	—	" "
—	" "	" " "	—	" "
—	*Bact. lep.* D ++ M Inf.	" " "	—	" "
—	" " " ++ " "	" " "	—	" "
—	" " " ++ " "	" " "	—	" "
—	" " M	" " "	—	" "
—	" " "	" " "	—	Chronic carriers
—	" " "	—	—	" "
—	" " D	*Bact. lep.* M	—	" "
—	" " "	" " "	—	" "
—	" " " + M Inf.	" " "	—	" "
—	" " " Inf.*bronch.*++	" " "	—	" "
—	*Bact. lep.* D+	" " "	—	" "
—	" " " +	" " "	—	" "
—	" " " G+	—	—	" "
—	" " D+++	—	—	" "
—	" . " " ++	*Bact. lep.* M	—	" "

TABLE III

No.	Fate	Clinical condition	Autopsy findings		
			Nasal passages	Ears	Lungs
C 99 F	Killed 12/21/26	Good	Normal	Normal	Normal
C 49 F	" "	"	"	"	"
C 79 F	" "	"	"	"	"
E 65 B	" 12/22/26	"	Pus	"	"
C 44 F–S	" "	"	"	Pus, left	"
C 21 F	" "	"	"	Normal	"
C 91 F	" "	"	Normal	Pus, bilateral	"
D 62 F–S	" "	"	"	" left	"
E 69 F–S	" "	"	"	" , "	"
E 77 F	" "	"	"	" "	"
C 46 F	" "	"	"	Normal	"
C 75 F–S	" "	"	"	"	"
D 44 F	" "	"	"	"	"
E 45 F	" "	"	"	"	"
E 75 B	" "	"	"	"	"
E 35 F	" "	"	"	"	"
E 28 F	Reserved	"	—	—	—
C 67 F–S	Killed 12/23/26	"	Normal	Pus, bilateral	Normal
C 92 F–S	" "	"	"	Normal	"
C 29 F–S	" "	"	"	"	"
E 97 F–S	" "	"	"	"	"
E 25 F–S	Reserved	"	—	—	—

Bact. lep. D = Rivers D type, instilled artificially 12/1/26.
Bact. lep. M = Mucoid type, spontaneous infection.
Bact. lep. G = Variant type, from Rivers D.
Inf. = Many colonies.

—Concluded.

Bacteriological findings				Group
Heart's blood	Nasal passages	Ears	Lungs	
—	*Bact. lep.* D+++	—	—	Chronic carriers
—	" " "++ G++	—	—	" "
—	" " M	—	—	" "
—	" " D+	—	—	Intermittent carriers
—	" " M Inf.	*Bact. lep.* M	—	"
—	" " D Inf.	—	—	"
—	*B. bronch.*	*Bact. lep.* M	—	"
—	D + *B. bronch.* Inf.	" " "	—	"
—	"++	D Inf.	—	"
—	*B. bronch.*	*Bact. lep.* M	—	"
—	*B. bronch.*Inf.*Bact.lep.*D+	—	—	"
—	*Bact. lep.* D+	—	—	"
—	*B.bronch.*Inf.*Bact.lep.*D+	—	—	"
—	*Bact. lep.* D Inf.	—	—	"
—	" " " "	—	—	"
—	*B. bronch.* Inf.	—	—	"
—	—	—	—	"
—	*Bact. lep.* D++	*Bact. lep.* M	—	Free
—	" " Inf.	—	—	"
—	*B.bronch.*Inf.*Bact.lep.*D+	—	—	"
—	" " " +	—	—	"
—	—	—	—	"

lesions and from the nasal passages of four. The Rivers D strain of *Bact. lepisepticum* was cultured from the nasal passages of eleven. Fourteen of the animals were classed as intermittent carriers. At autopsy pus was found in the nasal passages of three and in the middle ears of five. The mucoid form of *Bact. lepisepticum* was recovered from the ears of three; the D form from one. From the nasal passages of one mucoid colonies of *Bact. lepisepticum* were obtained; from nine, D forms. Five animals showed no *Bact. lepisepticum* in their nasal passages at any time during the experiment. At autopsy the nasal passages of all appeared normal. Pus was found in the middle ear of one. From here the mucoid form of *Bact. lepisepticum* was cultured. All four showed a few colonies of the Rivers D form.

When the animals are placed in these arbitrary groups, their differences in behavior appear the more striking. That some animals remained clinically healthy and free of infecting organisms during summer and winter, and at the time when temperature fluctuations and exposure were extreme, cannot be explained by chance. They were scattered amongst their companions, many of which were carriers, some snuffles cases, and still others ill with pneumonia. It seems more reasonable to regard the differences in behavior of these animals as due to innate and non-specific differences in their resistance.

Virulence.—At various times during the experiment, virulence titrations were made on strains of *Bact. lepisepticum* recovered from the nasal passages of various individual rabbits in the room. The technique of these tests has been discussed elsewhere, and the results of a number of them recorded in detail. The critical titrations were made by instilling equal doses of the various cultures into the nasal passages of young rabbits of similar age and weight, obtained from the breeding room. Subsequently, with these results as a basis, the mouse titration method was employed. This technique, although far more artificial, and of little or no value unless controlled by "natural" virulence titration, enabled us to examine a greater number of cultures and at more frequent intervals.

Many of these tests have been described previously.[2] In one instance[3] the results of intranasal titrations in rabbits of Cultures 544

[2] Webster, L. T., and Burn, C. G., *J. Exp. Med.*, 1926, xliv, 362, 366–376.

[3] Webster, L. T., and Burn, C. G., *J. Exp. Med.*, 1926, xliv, 362.

from Rabbit E 44 and 329 from Rabbit C 29 are summarized. In Tables IV, V, and VI the results of the intraperitoneal mouse titrations are shown. Culture 590 came from Rabbit E 90; Culture 560 was obtained on three different occasions from Rabbit E 60. Thus the strains in these tables may be identified: those in the 500's came from E rabbits; in the 400's from D rabbits; in the 300's from C rabbits, etc. These titrations emphasize two facts; first, that the form of *Bact. lepisepticum* brought into the room by the attendant and becoming endemic in the population was of the mucoid variety, similar to that found at the rabbit farm in New City, at Saranac, New York, and in other rabbits at the Rockefeller Institute; and secondly, that the virulence of all of these cultures proved equal and of moderate degree.

General Considerations.—The analysis of the part played by certain factors in the spread of *Bact. lepisepticum* throughout this population is rendered easier by reason of the control of disturbing influences. All the animals were exposed equally to the chance of infection so that theoretically those in the "complete" diet group should have been slightly more resistant than those receiving the "incomplete" ration. We assume that none of the rabbits had been previously exposed to the infection, and that none of the infecting organisms was in the room at the beginning of the experiment. We know that the infecting potentialities or virulence of the various strains of *Bact. lepisepticum* remained uniform throughout the entire period, and that the source of infection—attendants carrying organisms from infected rabbits in the neighboring room—remained constant.

At the outset, foci of infection were established in the nasal passages or middle ears of the rabbits naturally more susceptible, or rendered so by dietary deficiencies. During the succeeding 6 months available dosage was maintained at a level by chance contact with the attendant and by spread from local foci. Throughout the winter of 1925–26, procedures similar but more drastic than natural conditions were carried out to decrease further the resistance of the population. Dosage (carrier rate) increased rapidly, the weaker individuals succumbed to *Bact. lepisepticum* pneumonia and septicemia, and others developed local otitis media and snuffles. Still others, exposed to infection, remained refractory. As summer came on and the tem-

perature fluctuations were less severe, the population, reduced in numbers by the selective weeding our process, became more resistant, dosage fell, and cases of snuffles and numbers of deaths became less frequent.

In this experiment, therefore, factors influencing host susceptibility influenced the dosage of available bacteria and thereby determined the amount and severity of infection ensuing.

Control of Bact. lepisepticum Infection. Description of a Successfully Operated Rabbit Breeding Room.

As stated in the introduction, the purpose of this experiment was to test the conclusions derived from our studies of rabbit snuffles and pneumonia by attempting to keep a population of rabbits free of *Bact. lepisepticum* infection. The various procedures in this experiment were governed by two principles: first, the maintenance of host resistance at a maximum, and second, the decreasing of available dosage of *Bact. lepisepticum* to a minimum.

A special room was chosen in the midst of the space used for our normal rabbit stock. The room measures about 15 × 30 feet and has accommodations for thirty breeding females, ten males, and about fifty weaned young stock. Before bringing any animals into the room, the walls and floors were scraped carefully and washed with lysol, and the breeding cages were sterilized. The rabbits employed were selected from the normal stock obtained from various dealers within a radius of 200 miles of New York City. Such animals, upon their arrival at the Institute, were placed in isolation rooms, where they were observed for a period of 2 to 4 weeks. During this period any animals showing clinical evidence of snuffles were discarded.

During the spring and summer of 1923 several hundred of these animals were cultured from the nasal passages to detect the presence of *Bact. lepisepticum*. In view of the usual high percentage of carriers (1, *f*), a great many animals were examined before a suitable stock of breeders was obtained. No animal was admitted to the breeding room until three successive nasal cultures failed to demonstrate the presence of a single colony of *Bact. lepisepticum*. When an animal was finally admitted, its fur was sponged carefully with 5 per cent lysol. Thus by September a stock of about thirty females and ten males was accumulated.

The animals were cared for by an experienced attendant who has maintained a strict régime of cleanliness. In general, the door to the room is kept locked. Before entering the room, the attendant is careful to change his coat and wash his hands. The food is always taken from a fresh supply, and the animals are given a considerable amount of personal attention. The temperature in the room is regulated carefully at about 68°. The cages are cleaned every 2nd day.

The results of this experiment are striking, for by continuing the above careful methods of supervision we have maintained a breeding room entirely free of *Bact. lepisepticum* infection.[4]

At monthly intervals from September, 1923, to March, 1924, the entire breeding room was examined bacteriologically. Again, on September 24, 29, October 6, and December 17, each animal was cultured from the nasal passages. On March 18, 1925, twenty-two showed organisms of the *B. coli* group on hemolyzed blood agar plates streaked from cultures of the nasal passages. These organisms we have always considered as intestinal contaminants, occurring only at the external nares. Twelve showed *B. bronchisepticus*. These animals continued to be consistent carriers of this organism from the time they were selected for the breeding room until they died, or were discarded. None showed colonies of *Bact. lepisepticum*. On several occasions during 1926 young stock taken from this room have been cultured. On no occasion has *Bact. lepisepticum* been recovered. There have been no cases of pneumonia in the room, no clinical snuffles, no abscesses, and no otitis media.

The breeding record is summarized in Table IV. In it are tabulated: (1) the number of young weaned 2 months after birth; (2) the month of these weanings; (3) the number and date of sterile matings; (4) the date and number of times the young were destroyed; and (5) the fate of the various breeders. The males are not included in this

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[4] A few of the animals are affected with ear canker. This is treated in its early stages by local applications of tincture of iodine and lanolin. Likewise there is a certain amount of pinworm infection present, which can only be demonstrated at autopsy.

In 1924 some of the animals showed at autopsy the pathological changes characteristic of rabbit encephalitis. Clinically, the animals appeared perfectly healthy. No further diseases have been found.

TABLE

Rabbit No.	Sept., 1923	Oct.	Nov.	Dec.	Jan., 1924	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1925	Feb.	Mar.	Apr.	May
1					3				4			5							7		
2			6			2			M.										4		
3										6							4				
4			4					3	D.												
5			x					2		4					4		6		M.		
6								2 M.	D.												
7				4					3						5				3	D.	
8					2							5				M.		x		M.	D.
9								3	D.												
10									4							3				5	Dis.
11									5							x				4	
12									3							x				2	
13																4		x		x	
14												'		4	M.			2		M.	
15																	3		D.		
16																	5			x	
17																			3		
18												-					5			1	
19																	6	D.			
20															6					2:x	
21															3				1		
22															x		x	M.		M.	
23															3		x				
24															7				7		
25																x				2	Dis.
26																				4	
27																			1		x
28																x					
29																			1		
30																	M.				
31															x						4
32																	2				
33																			1		

D., signifies died; x, destroyed; Dis., discarded; M., sterile mating.

IV.

June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1926	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1927	Total
4				6	D.															29
	2	M.		Dis.																14
5							4	Dis.												19
																				7
	6			1	1			5						3	Dis.					32
																				2
																				15
																				7
																				3
																				12
		2	Dis.																	11
		1		1	Dis.															7
				3		x	Dis.													7
				1	Dis.															7
																				3
				2				3	M.				5	Dis.						15
		3			M.			M.	4				M.	M.	Dis.					10
		1					4			3		4			3					21
																				6
			3	D.																11
		6	x					8				5	Dis.							23
		1			2	Dis.														3
	2						2				5		4			3				19
3				5	M.					7	x				M.	Dis.				29
														.						2
3	M.			4						6			7		M.	M.	M.	M.		24
	M.						2	D.												3
2	M.	x					2	M.		6				2			4			16
4				5						8			1		M.			3		22
4	x	Dis.																		4
D.																				4
	1x			1			6	Dis.												10
6				5				8												20

TABLE IV—

Rabbit No.	Sept., 1923	Oct.	Nov.	Dec.	Jan., 1924	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan., 1925	Feb.	Mar.	Apr.	May
34																			6		
35																			D.		
36																			M.		
37																					6
38																					6
39																					1
40																			x		
41																				1	
42																			7		
43																					
44																					
45																					
46																					
47																					
48																					
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51																					
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60																					
61																					
62																					
63																					
64																					
Total.....		10	4	5	2	0	10	7	16	6	10	0	13	22	4	18		15	48	14	17

Continued.

June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan. 1926	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan. 1927	Total
3	x			5						4		5					5			28
	x			5						4			3		M.	M.	Dis.			12
4	M.	D.																		4
			2				5	M.			6		M.		M.	D.				19
	M.				5				7				7	M.	M.	M.				25
		2		x				4	M.				1		4	M.				12
		5				4					4				2	M.	M.			15
	1	D.																		2
	2				5	Dis.														14
	x		M.	M.				7					2		M.	M.		M.		9
	x		x					7			3				3	M.	M.			13
						4				5		3	M.				5			17
		x			M.				7	M.	Dis.									7
								7			1		M.		M.	Dis.				8
						4				2	D.									6
						3	M.			2			2		x	M.	Dis.			5
						3				2			2			3				10
					x					7				5	M.	M.	M.			12
								4	M.				x		1			x		5
										5	M.					6				11
											2M.				3			2		7
													2		M.	M.				2
											2				1			6		9
											1				4		M.			5
																2				2
															4					4
															2					2
																	4			4
																5				5
															2			4		6
																		4		4
38	13	19	6	26	33	18	41	42	18	45	33	32	21	10	28	15	19	23		701

table. The deaths recorded were due to parturition complications. None of these animals was infected with any microorganism. Animals were discarded for several reasons: first, for sterility or neglect of young, and secondly, for not conforming to the desired type. Dutch, Belgian, and albino crosses were specially selected because of their tendency to produce rapidly developing, small, heavy stock. During the year 1924, 95 young were weaned, an average of 8 per month and 3 per rabbit. In 1926, 265 were weaned, 22.2 per month, 9 per rabbit, and in 1926, 327 were weaned, 27.2 per month, 11 per rabbit.

The results of this experiment indicate that it is possible to maintain a stock of rabbits free from *Bact. lepisepticum* infection, although surrounded by groups in which the disease is widespread, by proper attention to the general condition or "natural" resistance of the animals, and by measures which minimize the available dosage of pathogenic microorganisms.

DISCUSSION AND SUMMARY.

In this paper we have attempted to describe the manner of spread of an endemic, native, respiratory infection and a method for its control. The essential factor determining the prevalence of such an endemic disease is, we believe, host susceptibility, which is controlled by hereditary and environmental influences. Furthermore, it seems probable that the amount of this population susceptibility determines the dosage of specific microbes available to the population.

An increase in dosage in the herd is followed by an increase in the spread and severity of the infection, and a decrease by a corresponding alleviation. Hence, two methods for the prevention of epidemics are available: (1) an enhancement of population resistance, and (2) the reduction to a minimum of available dosage. These procedures have proved successful for 3 years in maintaining a population of breeding rabbits, in the midst of a badly infected community, entirely free from *Bact. lepisepticum* infection.

Confirmation of the above conclusions has been gained from other studies in the field of experimental epidemiology. Dr. D. T. Smith (2), at Saranac, New York, found that changes in population susceptibility were responsible for a severe outbreak of *Bact. lepisepticum* infection and septicemia. Freund (3), at Berlin, has just published

an interesting account of respiratory epidemics of rabbits and guinea pigs, apparently brought about by sudden changes in temperature and housing conditions. Pneumonia and Pasteurella infection, endemic in the population, increased suddenly in extent and severity. Nevertheless, neither endemic nor epidemic strains of the microorganisms were found to be especially virulent. Dr. Theobald Smith (4), in a study of paratyphoid epidemics of guinea pigs, has made similar observations. He noted that pregnant females acted as the foci of infection, and that from these individuals, presumably of lessened resistance, the bacteria were given off and infection was spread.

The studies in experimental epidemiology are rapidly reaching a stage where they may be applied to the problems of human disease. Indeed, more recent observations of the mode of spread of pneumonia (5-7), scarlet fever (8), typhoid (9, 10), plague (11), diphtheria (12–14), measles (15), and tuberculosis (16–18) increasingly show a tendency to discard the theory of fluctuating microbic virulence and to emphasize the importance of the host factors.

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A THEORETICAL CONSIDERATION OF THE ACTION OF X-RAYS ON THE PROTOZOAN COLPIDIUM COLPODA.

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I. INTRODUCTION.

To show that the variation in reaction among the individuals of a group of apparently similar organisms fits a probability curve is, of course, not an end in itself, but rather a basis of conjecture as to the mechanism which provides the random element without which the theory of probability is meaningless. It is generally assumed that the reactions of various individuals differ because the individuals themselves are different. It is interesting, however, to consider the possibility that in some cases, where the organism is relatively simple, the individuals are essentially similar and the random element is inherent in the agent or in its primary effects. For example, if the agent is radiation, the quite generally accepted theory of absorption in *quanta* provides the necessary random element; and it may be that this is sufficient to account for the variations which we observe in the reaction to the rays of apparently similar simple organisms.

In his recent work on the action of x-rays on *Colpidium colpoda*, Crowther[1] finds that if he plots the number of survivors against the dose of radiation administered the result is a sigmoid curve; and, assuming the animals to be essentially alike, he shows that this is the kind of curve to be expected if the animal dies as a result of a certain number, n, of discreet events or "hits," all equally effective, and if the probability per unit dose of making a hit is constant. He finds, further, that theory agrees quantitatively with experiment when λ_e, the probability per e-unit of dose, is 5.9×10^{-4}, and n (for immediate death) is 49.

To account for this very small value of λ_e, he makes use of an inter-

[1] Crowther, J. A., *Proc. Roy. Soc. London, Series B*, 1926, c, 390.

esting hypothesis, which he advanced some time ago[2] for a similar purpose, that the effect is confined to some very small body or structure inside the animal. In doing so, he introduces certain difficulties, in my opinion. No general objection is raised against this hypothesis, which we may refer to as the "small-body theory." In this particular case, however, it appears to be inconsistent with the phenomenon which it was devised to explain, unless it is supplemented by certain rather novel assumptions as to the nature of the destructive effect of radiation on tissue. The nature of these assumptions will be brought out in what follows. An alternative explanation of the small value of λ_e which does not involve the small-body theory will also be suggested.

II. Primary and Secondary Effects of X-Rays.

The primary effect of x-rays on the light atoms of which living matter chiefly consists is the ejection of a high speed electron from some of them chosen at random in both space and time. Each of these primary electrons ionizes a large number of other atoms by collision before it comes to rest. This secondary ionization appears to be the only effect which we need consider. There is no good reason for supposing that the atom from which a primary electron has been ejected is the seat of any considerable part of the destructive effect; the disintegration of the particular molecule which contains this atom can scarcely be thought of as being more important, in general, than that of any other molecule. There is, on the other hand, plenty of evidence in favor of the view that the destructive effect is associated with the ionization produced by the high speed electron, in comparison with which the ionization by direct absorption of the rays is negligible. In very simple photochemical systems, the reactions produced by x-rays or by α- or β-rays proceed at a rate which is directly proportional to the rate of ionization; it would be rash, of course, to assert that this is true in the case of tissue destruction.

If the x-rays are monochromatic, the primary electrons are all ejected with the same speed and energy; they travel approximately equal distances before stopping, and they knock off about the same

[2] Crowther, J. A., Proc. Roy. Soc. London, Series B, 1924, xcvi, 207.

number of secondary electrons. The various quantum events or units are, therefore, much alike. Likewise, the events which consist in the production of the various secondary electrons are alike in one respect at least—all of the electrons have the same properties.

Doubtless one or the other of these units corresponds to the hit mentioned above. In what follows, I shall refer to the release of a secondary electron as an *electron-hit*, and to the emission of a primary electron with its attendant phenomena as a *quantum-hit*. The units of destructive effect dealt with in Crowther's analysis will be called *effective hits*.

III. Some Quantitative Estimates.

Crowther used the K radiation of molybdenum, the α lines of which have a mean frequency of 4.23×10^{18} per second. The primary electron is thus ejected with an amount of energy, $h\nu$, equal to 2.76×10^{-8} ergs. Dividing this by 5.5×10^{-11} ergs, the work required, on the average, to release a secondary electron in air according to Rutherford,[3] we find that each primary electron releases about 500 secondary electrons. This number will be denoted by E_q.

The fourth power law, together with Whiddington's[4] constant for air, shows that the maximum path length of the primary electron in air must be about .2 cm. The value taken directly from one of Sadler's[5] curves is .22 cm. Evidently the law holds nicely even for these very soft rays. In tissue, assumed equivalent to air of unit density, the maximum path, L, is, therefore, about 2.6×10^{-4} cm.

The paths of the primary electrons are, in general, not straight, and, in consequence, the distance in a straight line from the beginning to the end of the path is generally less than L. Consider a plane layer of air, the thickness of which, x, is uniform and somewhat less than L. If a great number of electrons enter this layer through one of its faces, all with the same speed, but in all possible directions, some of them will emerge from the opposite face with some part of their original energy. A fraction, then, of the energy which goes into the layer on one side comes out on the other side. Sadler[5] has shown that

[3] Rutherford, E., Radioactive substances and their radiations, Cambridge, 1913, 159.

[4] Whiddington, R., *Proc. Roy. Soc. London, Series A*, 1911–12, lxxxvi, 360.

[5] Sadler, C. A., *Phil. Mag., Series 6*, 1910, xix, 337.

the value of this fraction is given by $e^{-\mu x}$ in which μ is the mass-"absorption" coefficient of air for electrons of a particular initial speed. For molybdenum K electrons, he gives 1.18×10^4 as the value of μ. μ does not depend to any great extent on the nature of the absorbing material; we may say with safety that it has the same value for air and for tissue. This is the assumption on which we have already computed L.

The exponential law is, of course, not strictly true; it is inconsistent with the existence of a maximum path length. When the absorbing material reaches a thickness such that only those electrons which have travelled very nearly in a straight line can get through, a slight increase in the thickness will stop them all.

The distribution of secondary electrons along the path of the primary electron must now be considered. In any small part of the path, they are distributed very nearly at random—as nearly as the fine structure of matter permits. The space rate of ionization increases, however, as the velocity decreases. At the end of the path, the ionization is probably very intense. Glasson[6] states that over a considerable part of the path, at least, this rate (the number of electrons per cm.) varies inversely as the square of the velocity of the primary electron. This law may, of course, be derived directly from the fourth power law. On this basis, a simple calculation, which need not be given here, shows that by the time the primary electron which we are considering has reached the middle point of its path, it has released 150 secondary electrons, and that, at this point, the mean distance between consecutive electrons is about 7.3×10^{-7} cm. This is enough to show that we are not to think of the secondary ionization as being almost wholly confined to a small region near the end of the path.

Friedrich's e-unit of radiation is the amount required to release in 1 cc. of air at N.T.P. 1 electrostatic unit of charge of either sign, or 2.1×10^9 electrons. The mass absorption coefficient of tissue is the same as that of air and it is reasonable to assume, as Crowther does in his earlier paper, that the number of secondary electrons *per quantum* is the same. Since the tissue with which we are dealing is approxi-

[6] Glasson, J. L., *Phil. Mag.*, *Series 6*, 1910, xxii, 647.

mately of unit density, an e dose corresponds to the production of 1.63×10^{12} secondary electrons per cc. of tissue. This number will be called E_e.

IV. The Small-Body Theory.

That λ_e has been found to be very small shows that very few of the hits received by the animal, whether electron-hits or quantum-hits, are effective. From the fact that the atoms from which the high speed electrons are ejected are distributed at random in space, it follows that the probability that a high speed electron will be ejected from within any small portion of the animal is directly proportional to the volume of the portion considered and independent of its position. The same is true of the probability that a secondary electron will be released within the portion considered, provided, of course, that the volume is such that the electron-hits occur independently of one another. Assuming that the destructive effects are confined to some small body within the animal, we may assign volumes to this body such that either of these probabilities will assume any desired value,—in particular the value 5.9×10^{-4} in which case every hit within the small-body will be effective. On the hypothesis that the electron-hit corresponds to the unit of destructive effect, the diameter of the body (assumed approximately spherical) must be about 8.8×10^{-6} cm. Similarly on the quantum-hit hypothesis, the diameter is about 7.0×10^{-5} cm.[7]

Let us now inquire whether this theory is consistent with the postulates on which the statistical treatment of the problem is based. The postulates are: (1) that all effective hits are equally effective, and (2) that λ_e is constant.

Let us consider first the bearing of the electron-hit hypothesis on the small-body theory. L, the path length of the primary electron, is 29.4 times the diameter of the small-body appropriate to the hypothesis that the individual secondary electron corresponds to a unit of destructive effect. Since 500 such electrons are released by the primary electron in traveling a distance equal to L, it is evident that

[7] As the result of an error in calculation, which Dr. Crowther discovered after publication, the diameters assigned to the body in his paper differ somewhat from those given above.

in most cases, in which a high speed electron traverses the small body, it will release more than one secondary electron inside it. Those events, then, which are at random in time, are not the electron-hits at all, but rather showers of electron-hits, and the number of electrons per shower must vary within very wide limits, because the intensities of ionization, at the beginning and the end of the path, respectively, differ so much, and because the length of the path through the body ivaries from zero to the length of the diameter,—even more if the path is not straight. The average number of electrons per shower is, of course, very great in the case of primary electrons which enter from outside and come to rest inside the body; and correspondingly small for those which are ejected from within the body. For primary electrons which pass through the body, the average number of secondaries per shower is about 11, since the mean length of a great number of straight paths through a sphere, chosen at random, is equal to two-thirds of the diameter. Electron-hits are not then at random in time—not even approximately so—and postulate 2 is not fulfilled.

It is apparent then that we must abandon either the electron-hit idea or the small-body theory.

We have now to deal with the quantum-hit hypothesis in its relation to the small-body theory. Let us assume for the sake of the argument that the distribution of the destructive effect along the path of the primary electron is the same as that of the secondary ionization—which would be true if we were dealing with a simple photochemical system. It is evident that some of the primary electrons, ejected from atoms inside the small-body, must escape from the body with a considerable part of their initial energies. Likewise, other high speed electrons, ejected from matter outside the small-body, will enter it before coming to rest. In these cases, the effectiveness of the hit will be less than in the cases in which the whole path lies inside the body. Hits of this kind will be referred to in what follows as "partial" hits.

The relative number of partial hits cannot be so small as to be negligible. On the quantum-hit hypothesis, the diameter of the small-body is 7.0×10^{-5} cm., whereas L is 2.6×10^{-4} cm., i.e. 3.7 times the diameter. In order to make a very rough estimate of the relative importance of the partial hits, we set x equal to 3.5×10^{-5} cm., the

radius of the sphere, in the expression $e^{-\mu x}$ discussed in Section III, and we find that, of the energy associated with high speed electrons ejected from points midway between the faces of a layer of tissue of thickness equal to the diameter of the small-body, 66 per cent escapes from the layer. If we say that 66 per cent of the electrons escape, we shall make an underestimate, for each of the escaping electrons has lost a part of its initial energy. If we say that 66 per cent of the high speed electrons, ejected from the center of the small-body, escape, we shall underestimate the number still further, because the radius of the sphere is much smaller than the mean of the distances between a point in the middle of the plane layer and the points where the electrons escape from the surfaces of the layer.

Of the high speed electrons ejected from the center of the small-body, then, at least 66 per cent escape; of those ejected from points near the surface of the body, at least 50 per cent escape. Let us say that at least 50 per cent of all high speed electrons released within the small-body escape from it. Now for every one which escapes, another enters from outside. The whole number of hits, both total and partial, *i.e.* the whole number of those events which occur at random in time, is then increased by 50 per cent and two-thirds of them are partial hits. To keep the whole number down to 49, the body must be made smaller, and this will make the relative number of partial hits still greater. From what has been said in Section III about the distribution of ionization along the path it appears that we must give up either the small-body theory or the idea *that the distribution of the destructive effect along the path of the high speed electron is similar to that of the ionization.*

It is conceivable that the destructive effect, though brought about by ionization, is not measured by it; that it is conditioned in some way by the density of ionization or otherwise. It might be supposed, for example, that at the end of a path a small portion of tissue is injured so seriously that repairs are impossible; that at other points along the path the injury, being diffuse, is rapidly made good. If this were true and if the permanent injury which corresponds to an effective hit were confined to a very small region—to 1 per cent, let us say, of the path length, no objection could be raised against the small-body theory.

This idea, that the hit is localized in some very small part of the path, is the assumption referred to in the introduction. It should be noted that it amounts to something more than the assumption that some single molecule, peculiarly essential to the organism, happens to lie in the path of the primary electron and to be destroyed by it; if this were the case, the introduction of the small-body theory would no longer explain why all quantum-hits are effective and equally effective. Whether or not the difficulty of reconciling this assumption with the known facts of photochemistry is more than sufficient to compensate for the usefulness of the small-body theory is a matter of personal judgement.

V. An Alternative Hypothesis.

If the small-body were subdivided into a great number of much smaller bodies, and if these smaller bodies were placed as far apart as possible, the probability that a quantum would make two or more effective electron-hits would be made smaller. To make it negligible, however, the bodies would have to be very small in comparison with the mean distance between consecutive electrons in a shower. This suggests that an effective hit may correspond in some way with the destruction of molecules of a certain kind or kinds distributed throughout some considerable part of the tissue. The number, N, of such molecules present in the animal at the beginning of an exposure would have to be very great, of course, in comparison with n, which is 49, otherwise λ_e would become appreciably smaller as more and more effective hits were made. That the loss of so small a fraction of these molecules should have so profound an effect suggests either that they are essential parts of some structure or that the destruction of the molecule is followed by a recombination of the component atoms to form a molecule of a new substance which is highly toxic. The postulate that all effective hits must be equally effective seems to favor this latter idea, and to require that we restrict ourselves to one kind of molecule. We shall consider, then, that the making of a molecule of the toxic substance Y constitutes an effective hit. It appears highly improbable that the destruction of a molecule of substance X would always result in the production of a molecule of Y; it would certainly be more reasonable to suppose that Y is formed only when X loses

certain particular electrons. The problem cannot be analyzed, of course; too little is known about the fine structure of matter. In what follows, an attempt will be made to estimate the various quantities involved in the relatively simple case where Y is formed when X, represented by an idealized molecule, loses one particular electron. It will be assumed that in a microscopic sense the molecules of X are at all times distributed at random in space insofar as the finite size of the molecule permits,—the arrangement to be expected in a solution. It will appear further on that the molecule would have to be extremely large to have an appreciable effect on this distribution; it will be assumed tentatively that it has none.

Multiple Effective Hits Made by One Quantum.

Let us assume for the moment that, in the ordinary sense, X is distributed uniformly throughout the whole volume of the animal. If the probability that a quantum, falling entirely inside the animal, will make an effective hit be represented by p; and if V be the volume of the animal, then

$$p = \frac{\lambda_e \, E_q}{E_e \, V}. \tag{1}$$

V may be taken as 10^{-7} cc. Using the value of λ_e given by Crowther, and the values of E_q and E_e found in Section III, we find that $p = 1.8 \times 10^{-6}$. Now it is not the quantum as a whole, but rather the individual secondary electrons which correspond to the hits. In the language of probability we may, therefore, speak of the number of "trials" per quantum. If the molecule of X were so very small that it would never lose two or more electrons, the number of trials would be equal to E_q. If the molecule were larger, the number of trials would be less than E_q. When p is less than 1, a decrease in the number of trials, corresponds to a decrease in the ratio of p_r to p_1; p_r being the probability that the quantum will make exactly r effective hits and p_1 the probability of exactly one such hit. For example, if the number of trials were 1, the probability of a multiple hit would be absolutely zero. To find the maximum value of this ratio, which we may call R_r, we take the number of trials as infinite in which case R_r is $p^{r-1}/r!$ In particular, R_2 is $p/2$ or 9.0×10^{-7}.

R_r is so small that we may now reconsider the assumption that X is distributed uniformly throughout the whole volume of the animal. Other things being the same, p is proportional to the number of molecules of X per unit volume in the region where the quantum falls. If in some part of the animal the concentration of X were 1000 times as great as the mean concentration, then in this part p would be 1.8×10^{-3}; only about one effective hit in a million would be a "double," and one in less than 10^{12} a "triple" hit. p, for a particular quantum, cannot, of course, be greater than the value corresponding to the maximum concentration which the primary electron encounters; the concentration may change from point to point, therefore, as abruptly as desired.

It is evident, then, that effective hits are at random in time and that the molecules of X to be hit effectively are chosen at random, even though no unreasonable restrictions are placed on the way in which X is distributed.

The Size of the Molecule.

We have now to deal with the slow change in λ_e which takes place in consequence of the fact that N is finite. Let P_1 be the probability that a destroyed molecule of X, chosen at random from among the whole number of those that have been hit, will have lost exactly one electron; and let P' be the probability that a molecule, chosen at random from among all those which have lost exactly one electron, will have lost the particular electron required. In the normal case, the making of n effective hits corresponds to the destruction of n/P_1P' molecules of X, and, therefore, n/NP_1P' represents the relative change in λ_e. This latter quantity must then be small; just how small is a matter of judgment. It ought certainly to be smaller than the errors in experiment, and the results of Crowther's experiment fit the theoretical curve very nicely. The values of P_1 and P' depend on the properties of the molecule of X. As the volume, v, and the complexity of the molecule increase, both P_1 and P' diminish; furthermore, since Nv may not be greater than the whole volume of the animal, the maximum value which we may assign to N diminishes. The hypothesis is, therefore, consistent for a given value of v provided the corresponding value of NP_1P' is sufficiently large in comparison with

n and provided the value of P_1P' appropriate to a molecule of volume v is not so small that N has to be greater than V/v. It is obvious that these conditions are more easily fulfilled the smaller and simpler the molecule. We have to find out, if possible, whether or not they are fulfilled when the molecule is fairly large.

In order to estimate P_1 and P', it is necessary to make certain idealizing assumptions as to the nature of the molecule and to assign a definite size to it. To make it possible to treat P_1 statistically, it is assumed that the molecule will behave as though its electrons were distributed at random inside a sphere, the volume of which is the same as that of the molecule; the probability of releasing an electron being the same for all of them. For convenience, the diameter of the sphere is set equal to 10^{-7} cm. The volume is then equal to that of the molecule of oleic acid according to Langmuir.[8]

In the case of a complex organic molecule the electrons must be fairly evenly distributed throughout what we call its volume, *i.e.* the room which it occupies when stacked with other molecules to constitute matter in the solid state. Such a distribution, together with the movements of the electrons, and the random orientation of the molecule with respect to the path of the high speed electron may reasonably be thought of as equivalent to a random distribution. The probability that an electron will be released from the molecule is then directly proportional to the path length through the molecule. The constant of proportionality will be nearly enough equal to that for tissue in general, if we assign to the molecule the same number of electrons as that in the molecule of oleic acid, *i.e.* 158. P' will then be .0063. It will appear presently that, for a molecule of this size, the conditions imposed by the size of the animal and the desired constancy of λ_e are fulfilled with a margin of safety which is so great that the errors involved in idealizing the molecule need not be small.

We must now try to estimate P_1. Since the particular electron to be removed may be anywhere, we must suppose that it is in the worst place, *i.e.* at the center of the sphere. It will have the same chance of being hit wherever it is, but if it is at the center, the primary electron must traverse the longest path through the sphere to reach it,

[8] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

and the probability of removing two or more electrons from the same molecule increases with the path length. It has been shown in Section III that, on the average, 30 per cent of the ionization, *i.e.* 150 secondary electrons, lie in the first half of the path of the high speed electron, and that even at the midpoint of the path, consecutive secondary electrons are no closer together on the average than 7.3×10^{-7} cm., which is over seven times the maximum path length through our molecule. Let us confine our attention to the first half of the path for the moment. If the high speed electron were shot into a solid mass of X, the molecules being lined up in such a way that it would traverse a diameter of each, only about one molecule in seven at the midpoint of the path would lose an electron. When we remember that electrons are released farther and farther apart as we go from the midpoint toward the beginning of the path, we see that the number of cases in which a molecule loses two or more electrons must be very small in comparison with the number of those in which it loses only one. We shall make no great error if we assume that all hits in the first half of the path are "single hits." There are, undoubtedly, many single hits in the last half of the path, where the ionization is more intense, but we shall ignore them in order to make sure that we are not over-estimating P_1. The total number of single hits is then equal to 150, the number of electrons in the first half. It should be remembered that we assumed that the path follows the diameter of the molecule for the purpose of estimating the relative number of double hits. The number of single hits just found, 150, has nothing to do with the exact location of the path.

Now P_1 is, in the long run, the ratio of the number of molecules which have lost one electron to the total number destroyed by the loss of any number of electrons. For the average quantum, falling in a mass of X in the pure state, the whole number of molecules destroyed, which we will denote by M, must be less than 500, for some of the molecules lose two or more electrons. If all of the electrons in the last half of the path were lost by the same molecule, an absurd assumption, M would be 151, and P_1 would be 1. If all of the hits in the second half of the path were doubles, M would be 325 and P_1 would be .46. This is the minimum value of P_1. To sum up—P_1 lies somewhere between .46 and 1, and M lies between 150 and 500. Even

though the value of P_1 depends in part on M, we must consider the limiting values separately.

λ_e, the probability per e-unit of making an effective hit, is given by

$$\lambda_e = \frac{E_e}{E_q} \cdot N v M P_1 P'. \tag{2}$$

Substituting the limiting values of M, found above, we see that NP_1P' must lie between 6.9×10^5 and 2.3×10^6. To be on the safe side, we use the smaller of these numbers to test the constancy of λ_e. $n \div NP_1P' = 7.1 \times 10^{-5}$. In the normal case, λ_e may change, then, by as much as .007 of 1 per cent. Such a change is too small to consider.

Now we consider the maximum value of N. To make N as large as possible, we divide the greater value of NP_1P', which is 2.3×10^6, by the minimum value of P_1P', which is $.46 \times 6.3 \times 10^{-3}$, and N comes out to be 8.0×10^8. The total volume of X in the animal is then 4.2×10^{-13} cc., which is only 4.2×10^{-6} times the volume of the animal. The "volume" concentration of X is then only .0004 of 1 per cent; it is, of course, so small that cases will be very rare in which the finite size of the molecule interferes with the assumed random distribution.

The margins of safety in the variation of λ_e and in the total volume of substance X are obviously so great that the error involved in assuming that the real molecule behaves like the ideal one may also be very great without rendering the general hypothesis untenable. There can be little doubt that, if an effective hit corresponds to the removal of a particular electron from a molecule of X, the molecule of X may be fairly large and complex.

VI. CONCLUSION.

If we accept the idea that the reactions of living matter to x-rays are the result of ionization, we find that Crowther's small-body theory serves to explain the small value of λ_e only provided it be assumed further that the unit of destructive effect which corresponds to an effective hit is associated with the quantum and that it is localized in a region the dimensions of which are very small in comparison with the path length of the high speed electron.

It is suggested that there exists in the animal a substance, X, distributed throughout a considerable part of the tissue, a molecule of which turns into a molecule of a substance Y when it loses a particular electron, and that the formation of a molecule of substance Y constitutes an effective hit. This hypothesis seems to be consistent if the molecule of X is not too large. There is, of course, no good reason for supposing that it is the true explanation of the phenomenon. It is put forward merely to show that we may accept the theory that the variations in reaction are inherent in the x-ray itself without accepting the small-body theory.

VII. SUMMARY.

1. The theory which Crowther has advanced to account for the variation of the lethal dose of roentgen rays among the individuals of a group of *Colpidium colpoda* is reviewed.

2. It is shown that the use of his small-body theory to explain the small value of λ_s leads to certain further assumptions about the nature of the destructive effect.

3. An alternative hypothesis is discussed.

THE PREPARATION AND PURIFICATION OF LECITHIN.

BY P. A. LEVENE AND IDA P. ROLF.

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A revision of the methods of preparation and purification of lecithin has been undertaken for more than one reason. First, all the older methods employed in other laboratories as well as in our own are costly and time-consuming; second, judging from the numerous and frequent requests for the material which we have had from American and European colleagues, there seems to exist among biologists a considerable need for pure phosphatides.

The mode of purification employed in our laboratory has been continually modified depending upon the source and upon the condition of the material when it arrived at the laboratory. The procedure suggested in the present note is very simple and is applicable to all tissues. By it pure amino-free lecithin can be prepared within 24 to 48 hours.

However, the conditions for the preparation of the crude extracts may vary, depending on the starting material. The preparation of the extracts will be described separately for the three principal sources of lecithin; namely, for egg yolk, for brain, and for liver tissue.

Preparation of Crude Cadmium Chloride Salt of the Lecithin Fraction.

Egg Yolk.—Fresh egg yolks are made into a homogeneous emulsion by means of a mechanical stirrer (when such is lacking an ordinary egg beater can be used). The solution is strained through cheese cloth and poured into a double volume of hot 95 per cent alcohol. The alcoholic extract is allowed to cool and to it a cold saturated solution of cadmium chloride in methyl alcohol is added in quantity sufficient to produce a complete precipitation of the lecithin.

Liver Tissue.—40 pounds of fresh organs are freed from adhering adipose tissue, minced in a chopping machine, and dried. The dried tissue is extracted with 28 liters of 95 per cent alcohol. The alcoholic extract is concentrated to $\frac{1}{3}$ the original volume, and the solution is kept overnight in a refrigerator at 0°C. in order to allow the white matter to crystallize. To the filtrate a cold methyl alcoholic solution of cadmium chloride is added in quantity sufficient to complete the precipitation of lecithin.

Brain Tissue.—40 pounds of brain tissue are freed from adhering foreign tissue, minced in a chopping machine, and dried in a vacuum drying oven. The material is extracted twice with acetone (16 liters) and the residue is extracted with hot 95 per cent alcohol (24 liters). The further steps in the preparation of the crude cadmium chloride salt are the same as in the preparation of liver lecithins.

Purification of the Cadmium Chloride Salts.

Two conditions are essential to avoid decomposition of the lecithins in the course of their preparation. The first is to avoid high temperature when effecting solution or concentration and the second is to reduce to a minimum the use of water.

The principal impurity of the crude cadmium salts of lecithins is cephalin. In order to remove this substance the cadmium salts are well shaken with ether and the suspension is centrifugalized. The operation is repeated from eight to ten times. With the exception of the material extracted from liver, which still retains a yellow coloration, the cadmium salts are then perfectly white. At this stage, the cadmium salts contain very small proportions of cephalin. For further purification they are suspended in chloroform (400 cc. of the solvent for 100 gm. of the cadmium salts) and the suspension is shaken at room temperature until a slightly opalescent solution is formed. To this solution a cold 25 per cent solution of ammonia gas in dry methyl alcohol is added as long as a precipitate is formed. It is desirable to avoid a large excess of the reagent. The precipitate is removed by centrifugalization. The precipitate may be extracted with chloroform and the solution treated with methyl alcoholic ammonia.

The combined chloroform-methyl alcohol solutions of the lecithins are concentrated at about 10 to 15 mm. pressure, maintaining the water bath at 35–40°C. To obtain the residue in as dry a state as possible, it is dissolved in dry ether and concentrated to dryness under the same conditions as before. This operation is repeated three times. The final residue is extracted with 99 per cent alcohol. Should an appreciable proportion of cephalins be present in the lecithins, they remain as a residue insoluble in alcohol. Often, however, the residue is completely soluble in alcohol. This solution is treated with a methyl alcoholic cadmium chloride solution and the lecithins are liberated from cadmium chloride as before. The cadmium-free dry residue is dissolved in a minimum volume of ether and poured into acetone. 500 cc. of acetone suffice for the residue obtained from 100 gm. of the cadmium salts.

Further Purification of Lecithins.

The lecithins prepared in this manner form a light yellow mass and in the case of the brain, a practically white, semisolid mass. The material analyzes correctly for lecithin save for the nitrogen value which is generally slightly higher than 2 per cent. This high nitrogen value is due to small quantities of ammonia. The best procedure to remove this impurity is the following. 50 gm. of lecithin are dissolved in 50 cc. of ether. An equal volume of 10 per cent acetic acid is added and the mixture is shaken in a shaking machine for $\frac{1}{2}$ to 1 hour. A thick emulsion is formed which is poured into 500 cc. of acetone. The supernatant liquid is decanted and the precipitate is washed repeatedly with dry acetone. The wash acetone is added to the decanted water-acetone solution and the combined solution is concentrated under reduced pressure to dryness. The residue is dissolved in dry ether and the lecithin contained in it is precipitated by means of acetone. As a rule, the total loss of material is very small. The first, the purer material, contains about 50 per cent and the second about 25 per cent of the original lecithin.

Analytical.

Egg Lecithin.—Several samples of lecithin were prepared. The sample reported here had been reprecipitated as a cadmium chloride

salt seven times. In this case both the portion precipitated from the emulsion by means of acetone and the one obtained on concentration of the acetone solution analyzed correctly.

46.0 gm. of the material were dissolved in 50 cc. of ether and emulsified with 50 cc. of water. The emulsion was poured into 500 cc. of acetone. The precipitate contained 26.0 gm. of lecithin; from the mother liquor, on concentration, 10 gm. of lecithin were obtained.

The first 26.0 gm. were again emulsified and the lecithin precipitated with acetone. The yield this time was 22 gm.

Brain Lecithin.—The sample was reprecipitated with cadmium chloride six times. It was repurified by the acetic acid emulsion process only once (No. 170). The yield from 20 pounds of the brain tissue was 26 gm. On purification the yield was 19 gm. in the precipitate and 5 gm. were recovered from the mother liquor.

Liver Lecithin.—The material was reprecipitated with cadmium chloride three times. The yield was 50 gm. of lecithin from 40 pounds of fresh liver tissue. The material was purified with dilute acetic acid once.

The composition of the samples was as follows:

	C	H	N	$\frac{NH_2-N}{Total\ N}$	P	Iodine value.
Egg lecithin.						
First fraction (No. 134)..............	65.46	10.54	1.98	0	3.96	70
" " repurified (No. 152)....	66.01	10.59	2.03	0	3.90	62
Second fraction (No. 135)............	65.63	10.58	2.09	0	3.97	70
Brain lecithin (No. 170).	64.83	10.61	1.99	0	3.90	61
" " repurified (No. 171)......	65.53	10.69	2.00	0	3.90	47
Liver lecithin........................	64.83	10.19	2.24	0	3.99	90
" " repurified................	65.45	10.25	2.17	0	3.93	
Calculated.						
Oleic palmitic lecithin...............	64.81	10.81	1.80	0	3.99	32.7
" stearic "	65.55	11.01	1.73	0	3.85	31.5
Linolenic palmitic lecithin...........						98.1

CONFIGURATIONAL RELATIONSHIPS OF METHYLETHYL AND METHYLPROPYL CARBINOLS.

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The configurational relationship of dextro-methylethyl carbinol to dextro-lactic acid has been established.[1] The establishment of the relationship of the higher members of the homologous series of alcohols to the same reference substance will permit the correlation of the configurations of the alcohols among themselves. The present paper contains a report on the results of the efforts in this direction; namely, the results of experiments leading to conclusions regarding the configurational relationship of levo-methylpropyl carbinol to levo-lactic acid.

The task was facilitated by the fact that the configurational relationship of 4-hydroxyvaleric acid to lactic acid had already been established. Hence, it was necessary only to correlate 4-hydroxyvaleric acid with methylpropyl carbinol. The following set of reactions led to the desired end.

$$
\begin{array}{ccccc}
CH_3 & CH_3 & CH_3 & CH_3 & CH_3 \\
| & | & | & | & | \\
CHOH & CH\!-\!\!\!\rceil & CHOH & CHOH & CHOH \\
| & | & | & | & | \\
CH_2 & CH_2 & CH_2 & CH_2 & CH_2 \\
| \;\rightarrow & | \quad O \rightarrow & | \;\rightarrow & | \;\rightarrow & | \\
CH_2 & CH_2 & CH_2 & CH_2 & CH_2 \\
| & | & | & | & | \\
COOH & CO\!-\!\!\!\rfloor & CH_2OH & CH_2I & CH_3 \\
\text{Levo.} & \text{Dextro.} & \text{Levo.} & * & \text{Levo.}
\end{array}
$$
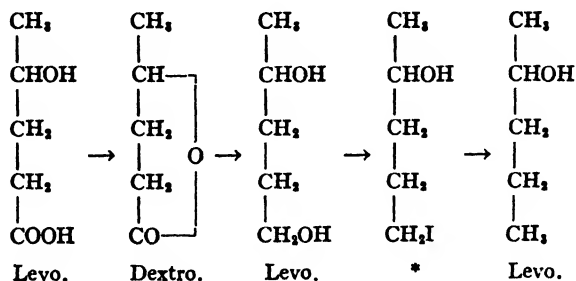
The methylpropyl carbinol was identified by means of its α-naphthylurethane. The rotation of the urethane was in the same direc-

[1] Levene, P. A., Walti, A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxi, 465.

* Rotation not determined.

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tion as that of the alcohol. In order to verify this conclusion, a naphthylurethane was prepared from dextro-methylpropyl carbinol obtained by resolution of the inactive carbinol. In the latter case also the carbinol and the urethane rotated in the same direction.

Thus it was found that the configuration of levo-methylpropyl carbinol is related to that of levo-4-hydroxyvaleric acid and hence to levo-lactic acid and, *vice versa*, dextro-methylpropyl carbinol to dextro-lactic acid. Inasmuch as dextro-lactic acid already has been correlated to dextro-methylethyl carbinol, it follows that dextro-methylethyl carbinol and dextro-methylpropyl carbinol are configurationally related.

In the course of the work with 1,3-dihydroxybutane it was found that on treatment with a halogen acid, the reaction product did not consist entirely of 1-halogen-3-hydroxybutane, but contained also a small proportion of 1-hydroxy-3-halogen butane. These two can be separated by fractional distillation. However, for a complete separation large quantities of starting material are needed. Fortunately, for the purpose of the present investigation, the separation of the two possible iodohydrins is not necessary as only glycols substituted in position (1) lead to optically active carbinols.

At this place mention may be made also concerning the stability of the <1, 4> ring in the amylene oxide. In the process of reduction of 1-iodo-4-hydroxypentane to methylpropyl carbinol, the oxide with a <1, 4> ring is formed as a by-product. It was found impossible to hydrolyze the oxide into the corresponding glycol, whereas ethylene or propylene oxide adds on a molecule of water rapidly.

<div align="center">EXPERIMENTAL.</div>

Dextro-γ-Valerolactone.—This lactone was obtained from the mother liquors in the resolution of γ-hydroxyvaleric acid with cinchonidine.[2] The solvent was removed by distillation under reduced pressure. The salt was dissolved in water and concentrated sulfuric acid was added until the reaction mixture was acid to Congo red. The solution was heated on a water bath for 15 minutes, cooled, filtered, and extracted with ether in a continuous ether extractor. The ethereal

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[2] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1926, lxix, 165.

extract was dried over anhydrous sodium sulfate and after removal of the solvent, the lactone was distilled. It boiled at 86–90°, p = 14 mm.

Levo-1,4-Dihydroxypentane.—Dextro-γ-valerolactone ($\alpha_D^{20} = +13.5°$ without solvent in 1 dm. tube) was reduced in 10 gm. lots with sodium and glacial acetic acid in the apparatus described by Levene and Allen.[3] 13.8 gm. of sodium were emulsified in 100 cc. of dry toluene and 10 gm. of the lactone dissolved in 20 cc. of glacial acetic acid were then introduced. The solution was added at such a rate that the introduction required 8 minutes. During the course of the reaction, 100 cc. of toluene were gradually added through the condenser. After the addition of the lactone, 16 cc. of glacial acetic acid in 20 cc. of toluene were added followed by 25 cc. of absolute alcohol when refluxing had ceased. The reaction mixture was cooled and filtered. The filtrate was concentrated under reduced pressure. To the residue absolute alcohol and ether were added, the precipitate was filtered off, and the filtrate, which was acid to litmus but not to Congo red, was concentrated on the water pump to a thick syrup. This was distilled on a high vacuum pump. The glycol distilled at 95–96°C., p = 1.5 mm.

0.1404 gm. substance: 0.2974 gm. CO_2 and 0.1458 gm. H_2O.
$C_5H_{12}O_2$. Calculated. C 57.70, H 11.54.
Found. " 57.76, " 11.62.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{22} = \frac{-1.55° \times 100}{1 \times 31.6} = -4.90°.$$

55 gm. of lactone yielded 44 gm. of the glycol. 30 gm. of lactone ($\alpha_D^{20} = +10.25°$ without solvent in 1 dm. tube) were reduced in the same manner as described above and yielded 24 gm. of glycol. In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{-1.05° \times 100}{1 \times 28.3} = -3.70°.$$

Di-(Phenylurethane) of Levo-1,4-Dihydroxypentane.—1 part of 1,4-dihydroxypentane ($[\alpha]_D^{20} = -4.90°$) and 2.4 parts of phenyl-

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[3] Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 443.

isocyanate were heated on a steam bath for 1 hour. The reaction mixture was then allowed to stand overnight. The viscous mass was washed with petrolic ether and crystallization was induced with a stirring rod. After washing with petrolic ether, the urethane was recrystallized from 95 per cent alcohol. It melted at 131–133°C. and analyzed as follows:

0.0997 gm. substance: (Kjeldahl) 5.80 cc. 0.1 N acid.
$$C_{19}H_{22}O_4N_2. \quad \text{Calculated.} \quad \text{N } 8.18.$$
$$\text{Found.} \quad \text{``} \quad 8.14.$$

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{-0.11° \times 100}{4 \times 3.87} = -0.71°.$$

1-Iodo-4-Hydroxypentane.—For the preparation of this substance, the combined glycols obtained in the preceding experiment were employed. Into the glycol slightly more than 1 equivalent of dry hydrogen iodide was passed, the reaction mixture being maintained at 0°C. It was then heated on the water bath for 1 hour, cooled, ice and chloroform added, and the mixture neutralized with sodium carbonate. The chloroform extract was dried over sodium sulfate. After removal of the chloroform the remaining iodohydrin was reduced directly without further purification.

Levo-Methylpropyl Carbinol.—The iodohydrin obtained in the foregoing experiment was reduced with hydrogen in the presence of colloidal palladium. The procedure was the same as that described[1] for the reduction of 1-iodo-3-hydroxybutane.

The ether was distilled off, using a fractionating column, and the residue was distilled.

Fraction   I.   80–84° 8 gm. α = −2.18° in a 1 dm. tube.
   "   II.   85–116° 3 "
   "   III.   116–120° 4 " α = −1.20° " " 1 "   "

Fraction I consisted chiefly of pentylene oxide.[4]   Fraction III had the boiling point of methylpropyl carbinol. It was therefore redistilled and converted into the α-naphthylurethane.

α-Naphthylurethane of Methylpropyl Carbinol.—1 part of the alcohol

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[4] von Ehrenthal, B. P., *Monatsh. Chem.*, 1903, xxiv, 354.

obtained in the above experiment and 2 parts of α-naphthylisocyanate were heated on the steam bath for 15 minutes. The reaction mixture was allowed to stand overnight. It was then washed with petrolic ether and extracted with hot absolute alcohol. To the alcoholic filtrate water was added until an oil separated which readily solidified on cooling. It was recrystallized from dilute alcohol. It melted at 71–73°C. and analyzed as follows:

0.0500 gm. substance: (Kjeldahl) 1.93 cc. 0.1 N acid.
$$C_{16}H_{19}O_2N. \quad \text{Calculated.} \quad N\ 5.44.$$
$$\text{Found.} \quad \text{``} \quad 5.40.$$

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{20} = \frac{-0.42° \times 100}{2 \times 7.5} = -2.8°.$$

The above results were substantiated by converting dextro-methylpropyl carbinol into its α-naphthylurethane. The carbinol was obtained by the resolution of inactive methylpropyl carbinol as described by Pickard and Kenyon.[5]

The carbinol without solvent had a rotation of $\alpha_D^{20} = +7.65°$ in a 1 dm. tube. The α-naphthylurethane was prepared in the usual manner. It melted at 88–91°C. In absolute alcohol it had the following rotation.

$$[\alpha]_D^{21} = \frac{+0.65° \times 100}{1 \times 4.9} = +13.3°.$$

It analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 3.90 cc. 0.1 N acid.
$$C_{16}H_{19}O_2N. \quad \text{Calculated.} \quad N\ 5.44.$$
$$\text{Found.} \quad \text{``} \quad 5.46.$$

[5] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 64.

ACETYL MONOSES.

III. ON α-MANNOSE PENTACETATE.*

BY P. A. LEVENE AND ISAAC BENCOWITZ.

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The ring structure of free monosaccharides and of their derivatives has been the subject of considerable disagreement. It naturally follows that either the postulates of individual authors are not always correct or that the substances discussed by them are not always correct. The purity or homogeneity of the derivatives of mannose particularly has been questioned. Regarding the so called α-pentacetate of mannose, Hudson[1] writes as follows: "For the present the acetate of + 55 rotation will be left unclassified; the determination of its ring form and even the question whether it may not be a mixture of substances remain outstanding problems." It is, however, peculiar that the values for $[M]_{D\alpha} - [M]_{D\beta}$ for the two known pentacetates of mannose are the same as for the two common forms of pentacetates of galactose and on this basis Levene and Sobotka[2] are inclined to

* The first two papers of the series on monose pentacetates contained several errors, which were kindly called to our attention by Dr. C. S. Hudson.

Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926, lxvii, p. 766, line 16 should read "have been prepared by Dale from methylmannoside and from non-crystalline bromotetracetylmannose." From the crystalline bromo derivative the γ-form of Dale was prepared by us.

On p. 768 line 8 from the bottom should read, "causes a change from levo- to dextrorotation."

On p. 774 foot-note 4, instead of "acetobromomannose" should read "acetochloromannose."

Also, it is regretted that an article by Dr. C. S. Hudson and J. K. Dale in which mention is made of two crystalline methyltetracetylgalactoses had been overlooked by us. It is unfortunate that these two substances were never described in detail and therefore not recorded in *Chemical Abstracts*.

[1] Hudson, C. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1433.

[2] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926, lxvii, 759, 771.

attribute to these four pentacetates the $<1, 5>$ ring structure.   It is realized, however, that the pentacetate of $+55°$ rotation has hitherto been crystallized with great difficulty and was never recrystallized with sufficient rigor and that therefore the skepticism of Hudson regarding its purity is justified.

The method of preparation of the mannose pentacetate has now been improved and it has been found possible to recrystallize the substance nine times without changing its optical rotation or its melting point.   The latter, however, has been raised from $64°$ to $75°C$. and after reaching that point, it remained constant.   Thus, there seems to be little doubt as to the purity of the mannose pentacetate of $+55°$.

Inasmuch as the explanation of the irregular optical behavior of mannose and its derivatives is of great importance for the further development of the work on the relationships of structure and optical rotation in the sugars, it was concluded to test the optical dispersion of α-mannose pentacetate.

The rotatory dispersion of homogeneous substances generally exhibits a normal course.   Biot classified as normal such dispersions as have the locus of the specific rotations in monochromatic lights on a straight line.   He regarded as abnormal the dispersion in which the curve connecting the specific or molecular rotations as a function of the wave length has a maximum.   Drude later gave mathematical expressions for the dispersion curves of different substances.   In certain cases the molecular dispersion could be represented by the expression

$$[M] = \frac{K}{\lambda^2 - \lambda_0^2}$$

In more complicated cases the course is given by the expression

$$[M] = \frac{K_1}{\lambda^2 - \lambda_1^2} + \frac{K_2}{\lambda^2 - \lambda_2^2} + \frac{K_3}{\lambda^2}$$

Lowry referred to the first form of rotatory dispersion as "simple" and to the latter as "complex."

Biot recognized that the most common cause of irregular dispersion is the non-homogeneous composition of the material of which the dispersion is measured.   Hence, there is always a doubt in regard to the

homogeneous character of a substance if it exhibits an irregular or complex dispersion. On the other hand, a simple dispersion speaks in a measure in favor of homogeneity.
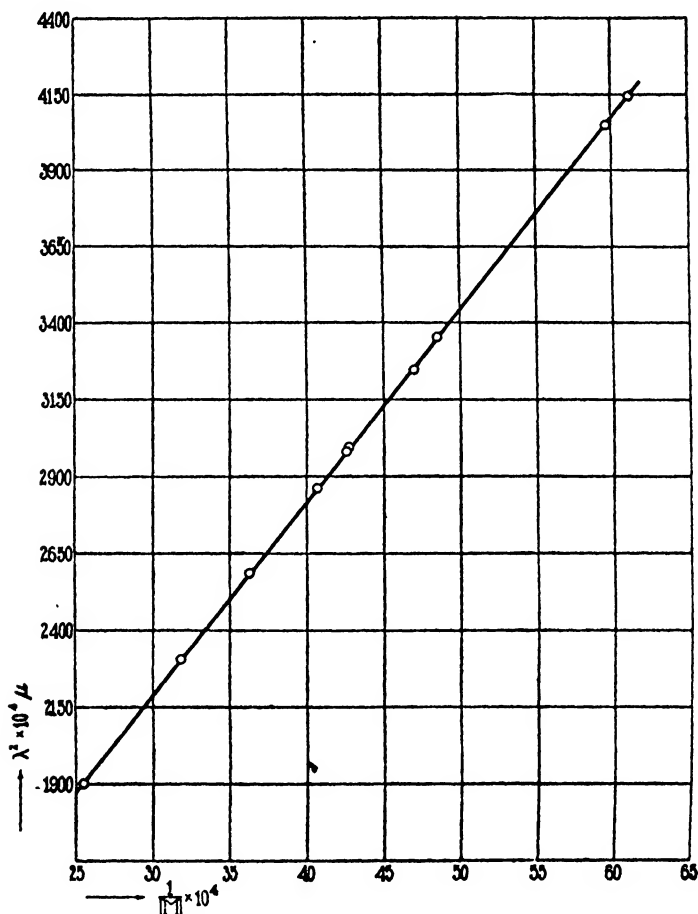
FIG. 1. Optical rotatory dispersion of α-mannose pentacetate.

The results of the observations on α-mannose pentacetate are plotted in Fig. 1, where the abscissas represent the reciprocal of the molecular rotations and the ordinates represent the squares of the wave-lengths. It is seen that the experimental points lie on a straight line. This establishes the accuracy of the experimental technique

and indicates that the dispersion of α-mannose pentacetate can be expressed by one term of Drude's equation. This fact is more strikingly shown in Table I where in Columns 6 and 7 are given the observed and calculated molecular rotations respectively. The calculated molecular rotations were obtained by means of the equation:

$$[M] = \frac{K}{\lambda^2 - \lambda_0^2}$$

TABLE I.

Optical Rotations of α-Pentacetyl Mannose for Ten Wave-Lengths.

$c = 1.174$ mols per 1000 cc. $\lambda_0^2 = 0.030$

$t = 25° \pm 0.1°$ $K = 7985 \pm 10$

(1)	λ (2)	α (3)	Average deviation. (4)	$[\alpha]^{25}$ (5)	[M] observed. (6)	[M] calculated. (7)	Difference. (8)
			per cent				*per cent*
Cd red..............	6438	19.19	0.08	41.90	163.3$_9$	163.5$_8$	−0.12
Zn "	6364	19.67	0.10	42.94	167.4$_8$	167.7$_4$	−0.15
Cu yellow..........	5790	24.16	0.06	52.75	205.7$_1$	206.0$_1$	−0.15
Hg "	5700	24.97	0.08	54.51	212.6$_1$	213.2$_5$	−0.30
Ag green...........	5472	27.44	0.07	59.91	233.6$_4$	233.4$_8$	+0.08
Hg "	5461	27.60	0.06	60.26	235.0$_0$	234.5$_0$	+0.21
Tl "	5351	28.86	0.07	63.01	245.7$_3$	245.4$_1$	+0.15
Cd "	5086	32.36	0.07	70.65	275.5$_3$	275.0$_6$	+0.17
Cd blue............	4800	36.85	0.09	80.45	313.7$_6$	313.8$_0$	−0.01
Hg violet..........	4359	46.08	0.20	100.6	392.5$_3$	393.0$_0$	−0.12

where [M] is the molecular rotation, λ^2 is the square of the wave-length and K and λ_0^2 are constants. The differences of the two values given in Column 8 are not only within experimental error but are irregular in magnitude and sign, which establishes the fact that one term of Drude's more general equation:

$$[M] = \Sigma \frac{K}{\lambda^2 - \lambda_0^2}$$

is sufficient to reproduce the experimental dispersion curve (within our range of wave-lengths and within the experimental precision).

In Column 3 are given the average rotations, in Column 4 are the average deviations of the mean to ten readings.

EXPERIMENTAL.

A. Purification of α-Mannose Pentacetate.

Pentacetate of mannose was prepared in the manner previously described[3] by the action on mannose of acetic anhydride in the presence of pyridine. The reaction product is poured over a mixture of chopped ice and very little water and the mixture is vigorously stirred. The water is removed from time to time until finally the pentacetate is turned into a semisolid mass. This product is then dissolved in chloroform, washed free from pyridine, and the final chloroform solution is dried first with calcium chloride and subsequently with anhydrous sodium sulfate. The dried solution is evaporated nearly to

TABLE II.

Results of Recrystallization.

All rotations were made in chloroform solution.

$l = 1.00$ dm. $c = 2$ per cent. $t = 22°C.$

	$[\alpha]_D$	M.p.
Crude material..	45°	56
Recrystallized out of 40 per cent CH₃OH...................	50	64
" " " 30 " " "	50	67
" " " 25 " " "	52	72
" " " ether.............................	54	75
After ninth recrystallization.............................	55	75

dryness-under reduced pressure, the temperature of the water bath being 40°C. The residue is then taken up in ether and again concentrated nearly to dryness. The final residue is dissolved in a great excess of anhydrous ether and placed in a desiccator over phosphorus pentoxide and paraffin. Slowly in the course of 24 hours a crystalline deposit is formed. If the original mannose was the α form, containing some of its isomer, the first deposit is the nearly pure β-pentacetate. This deposit is filtered off and the mother liquor is returned to the desiccator. The pentacetates remaining in solution are allowed to crystallize fractionally. The crystalline deposits are

[3] Levene, P. A., *J. Biol. Chem.*, 1924, lix, 141.

filtered off at 24 hour intervals. If the proportion of the β form is already small, the second fraction may consist in the main of the so called α form. Such crystallizations have been carried out many times. In the experiment to be recorded here, the second crystalline deposit consisted in the main of the α form. Its rotation was $+48°$ and it melted (not sharply) at 58–60°C. This substance was recrystallized at first out of 40 per cent methyl alcohol, subsequently out of 30 per cent methyl alcohol, and finally out of 25 per cent methyl alcohol. The first crystallizations proceeded very slowly so that between 24 and 48 hours were required for completion of the crystallization. The final crystallizations were completed so rapidly that several recrystallizations could be made within 1 day.

Table II contains the melting points and the rotations of the substances obtained by successive recrystallizations.

B. Procedure for Measuring Rotatory Dispersion.

All measurements were made with a triple field Schmidt and Haensch polarimeter provided with a large direct vision spectroscope.

The rotations of the solutions were determined in jacketed tubes. A rapid flow of water at 25°C. maintained a constant temperature. Thermometers divided into tenths of a degree were placed at the inlet and outlet of the tubes. At no time during a run was there a variation of temperature of more than ±0.1°C.

Sources of Light.—The mercury green, 5461 Å, and the violet, 4358 Å, lines were obtained from a mercury arc. The light of this lamp was purified by the spectroscope and was tested by means of a quartz test plate which was recently calibrated at the Bureau of Standards. This test served as a check on the purity of the light source; *i.e.*, the efficiency of the spectroscope, the effect of possible stray light, and the accuracy of the polarimeter. In every case the rotation of the respective lights by the quartz plate checked the rotations obtained by the Bureau of Standards within the precision limit of our instrument (±0.02°).

The Cd, Zn, Cu, Ag, and Tl lines were obtained in the following manner. The light of a small incandescent bulb with a horizontal tungsten filament was focused upon the collimator slit of the spectroscope. The light after having passed the prism, the slit at the tele-

scope end, and the polarizing Nicol prism was rotated by means of the quartz test plate.

The theoretical rotations of our test plate for ten monochromatic lights were calculated. The data of Lowry[4] for the rotation of light in quartz were used. The prism of the spectroscope was then adjusted until the patch of light which passed through the polarimeter was rotated the proper amount. The wave-length of the optical center of this patch was assumed to be the same as that of the monochromatic light which gave a corresponding rotation in the quartz test plate.

TABLE III.

Comparison of Rotations of the Green Mercury Line 5461 Obtained from the Mercury Arc and That Obtained from a Continuous Spectrum by Means of a Quartz Test Plate.

Concentration mols per 100 cc. (1)	α 5461 arc. (2)	α 5461 continuous. (3)	Difference. (4)
			degrees
0.1987 ·	10.20	10.18	0.02
0.4016	10.32	10.29	0.03
0.6124	15.30	15.30	0.00
1.174	27.63	27.60	0.03
2.060	47.16	47.18	0.02

To test the validity of this assumption, especially when the light was rotated by a solution which had a different dispersion curve than that of quartz, the following simple experiment was carried out. Green light from the arc, tested by means of the quartz plate, was passed through the polarimeter and was rotated by several solutions of various strengths. Then the arc was replaced by the incandescent lamp and the spectroscope adjusted until the patch of light illuminating the polarimeter gave the same rotation in the quartz test plate as the light from the arc. The test plate was then replaced by the various solutions and the rotations obtained with this light from the continuous spectrum of the incandescent lamp were compared with the rotations obtained with the monochromatic light from the arc. The

[4] Lowry, T. M., *Tr. Phil. Soc.*, 1912, ccxii, 261.

results are given in Table III.   The rotations of the green light from the arc are given in Column 2; those of the patch of light from the continuous spectrum are given in Column 3.   The differences between the two are given in Column 4.   It is seen from these figures that the agreement is remarkable.   Additional corroboration of the validity of our assumption is obtained from the results shown in Fig. 1, and given in Table III.

Manipulation.—The zero point of the tube filled with the solvent was taken for each wave-length.   The tube was then emptied, dried, and refilled with the solution.   Before each reading the spectroscope was set by means of the quartz test plate so that the optical center of the patch illuminating the polarimeter had the desired wave-length.   At least ten readings for each wave-length were taken.   At the end of the run, the spectroscope was readjusted for the various wave-lengths and five additional readings for each wave-length were taken.   This served as a check on the setting of the monochromator and indicated any change in concentration which might have taken place during the run.

THE EFFECT OF IONIZATION UPON OPTICAL ROTATION.

II. RELATIONS IN THE SERIES OF AMINO ACIDS, POLYPEPTIDES, AND KETOPIPERAZINES.

BY P. A. LEVENE, LAWRENCE W. BASS, ROBERT E. STEIGER, AND ISAAC BENCOWITZ.

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It has long been known that for any given optically active electrolyte the ion and the undissociated molecule generally exhibit individual and characteristic optical rotations. The change in rotation due to a change in the equilibrium between ions and undissociated molecules can therefore be employed in measuring the degree of dissociation of an optically active electrolyte as a function of external conditions, such as hydrogen ion concentration. The method can likewise be applied to the determination of dissociation constants and it should prove to be of particular interest in the case of dissociation constants which lie in ranges in which the electrometric method no longer permits accurate measurement.

It has recently been shown by Levene, Simms, and Bass[1] for nucleotides that a plot of molecular rotation [M] against corrected equivalents of base b' gives straight line curves between the integral values of b'. Also Wood,[2] Vlès and Vellinger,[3] and Vellinger[4] have investigated the relation between optical rotation and ionization.

In the present paper the method has been applied to a study of enolization in peptides and ketopiperazines.

The phenomenon of enolization in proteins has been assumed by

[1] Levene, P. A., Simms, H. S., and Bass, L. W., *J. Biol. Chem.*, 1926, lxx, 243.

[2] Wood, J. K., *J. Chem. Soc.*, 1914, cv, 1988.

[3] Vlès, F., and Vellinger, E., *Compt. rend. Acad.*, 1925, clxxx, 742.

[4] Vellinger, E., *Compt. rend. Acad.*, 1926, clxxxii, 1625.

many authors. This enolization was generally thought to occur in the imide group:

$$H_2N \cdot CH\text{---}CO\text{---}NH\text{---}CH \cdot COOH \rightleftharpoons H_2N \cdot CH\text{---}C\text{==}N\text{---}CH \cdot COOH$$
$$\underset{R_1}{|} \qquad\qquad \underset{R_2}{|} \qquad\qquad\qquad \underset{R_1}{|} \ \underset{OH}{|} \quad \underset{R_2}{|}$$
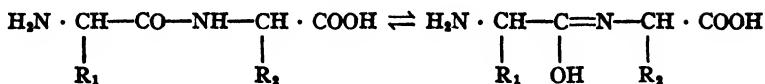
Dakin[5] was the first, to our knowledge, to suggest still another form of enolization involving 2 carbon atoms:

$$H_2N \cdot CH\text{---}CO\text{---}NH \cdot CH \cdot COOH \rightleftharpoons H_2N \cdot C\text{==}C\text{---}NH \cdot CH \cdot COOH$$
$$\underset{R_1}{|} \qquad\qquad \underset{R_2}{|} \qquad\qquad\qquad \underset{R_1}{|} \ \underset{OH}{|} \qquad \underset{R_2}{|}$$
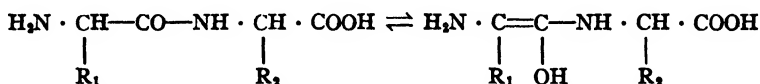
According to Dakin, this enolization takes place in peptides only when the chain contains at least three amino acids. Levene and Pfaltz[6] have recently shown that enolization of this type takes place more readily in ketopiperazines and to a less degree in polypeptides. Thus it is possible that in polypeptides the first type of enolization predominates, whereas in ketopiperazines the second type prevails. Enolization of either type, however, leads to the formation of an additional ionizable group of which the degree of dissociation should be measurable by the polarimetric method.

A study of dextro-alanyl-dextro-alanine anhydride was made first, in view of the fact that this substance contains no free carboxyl or amino groups; hence, any buffer effect produced by it may justly be attributed to enolization. Indeed, E. Fischer, having observed the ready hydrolysis of ketopiperazines by alkali, assumed an intermediate phase of salt formation due to enolization. Euler and Pettersson[7] have recently expressed a similar view and they estimated a value for the dissociation constant of glycyl-glycine anhydride of the order of magnitude of 10^{-15}. By means of the polariscopic method we have determined the dissociation constant of dextro-alanyl-dextro-alanine anhydride with an approximate value of $10^{-13.5}$ (pG′ = 13.5 ± 0.1).

The method was then applied to a tetrapeptide, glycyl-levo-

[5] Dakin, H. D., *J. Biol. Chem.*, 1912–13, xiii, 357.

[6] Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxiii, 661; *J. Gen. Physiol.*, 1925, viii, 183; *J. Biol. Chem.*, 1926, lxviii, 277; 1926, lxx, 219.

[7] Euler, H., and Pettersson, E., *Z. physiol. Chem.*, 1926, clviii, 7.

alanyl-levo-alanyl-glycine. This substance showed a considerable change in rotation when the hydrogen ion concentration was reduced to pH 13.35 by the addition of 5 mols of alkali. It is unfortunate that lack of material prevented us from making more than one measurement in the third buffer range. However, the fact that the two dissociation constants (due to the carboxyl and amino groups) of the tetrapeptide are of the same order of magnitude as those previously determined for amino acids and simpler peptides warrants the conclusion that the third change in rotation is due to one or more additional dissociation constants, namely the enolic dissociation constants.

In order to test whether this change might have been due to the effect of a high concentration of alkali, the titration-rotation data were determined for dextro-alanine. In this case no abnormality was observed in the data; *i.e.*, no further changes in rotation were observed when the pH was raised far beyond the range of the second dissociation constant.

From the data given in the experimental part it is seen that the agreement between the electrometric and polarimetric values of the degree of dissociation is such as may be expected from the precision of our polarimeter, which is accurate to ±0.02°. With an apparatus of greater precision we hope to obtain very accurate values of ionic concentrations.

EXPERIMENTAL.

1. Preparation of Compounds.

Dextro-alanine was obtained by the hydrolytic products of silk by the procedure described by Fischer.[8]

Dextro-alanyl-dextro-alanine anhydride was prepared from dry dextro-alanine ethyl ester.[9] 46.9 gm. were heated 24 hours in an oil bath at 100°C. The crystals of anhydride which separated were filtered off by suction and were then washed repeatedly with ether. The yield was 5 gm. (17 per cent of the theory). The product was purified by recrystallization from 10 times its weight of boiling water.

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[8] Fischer, E., *Ber. chem. Ges.*, 1906, xxxix, 462.

[9] *Cf.* Fischer, E., *Ber. chem. Ges.*, 1906, xxxix, 468.

Glycyl-levo-alanyl-levo-alanyl-glycine was prepared according to the directions of Levene and Pfaltz.[10]

2. Polarimetric and Potentiometric Measurements.

Preparations of Solutions.—All solutions were prepared from analyzed samples and the respective concentrations were corrected on the basis of the analyses.

Measurement of Rotations.—The rotations were measured at 25°C. for the wave-length λ = 5461 Å, obtained from the light of a mercury arc by purifying it with a direct-vision spectroscope, in 2.00 dm. open tubes (with ground glass covers) containing 4 cc., or in 4.00 dm. open tubes (with glass plate covers) containing 22 cc.

The values recorded in the tables represent the mean of a series of at least six readings.   The accuracy of the polarimeter was ±0.02°. The relative values, however, were much better.

Measurement of pH Values.—The pH measurements were made at 25°C. in water-jacketed hydrogen electrode cells.   The pH value 1.075 of 0.1000 N HCl was used as a standard.   The saturated KCl liquid junction was assumed constant.

3. Calculations.

Titration Data.—The calculations from the titration data were made by the method described previously,[11] the following equations being employed:[12]

$$ph = \text{pH} - \log \tau_{\text{H}} \tag{1}$$

$$poh = (13.89 - \text{pH}) - \log \tau_{\text{OH}} \tag{2}$$

$$b' = \frac{b-a}{c} + \frac{h-oh}{c} \tag{3}$$

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[10] Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1926, lxx, 219.

[11] Levene, P. A., Bass, L. W., and Simms, H. S., *J. Biol. Chem.*, 1926, lxx, 229. Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

[12] The notation is the same as that employed in previous papers except that γ is used for degree of dissociation instead of α to avoid confusion with α denoting rotation.

where H refers to activities and h and oh to concentrations. The values of pG′ were obtained from the relation

$$pG' = pH - \log \frac{\gamma}{1 - \gamma} \qquad (4)$$

Rotation Data.—The theory of the calculations from the rotation data is discussed in a previous paper.[18]

The degree of dissociation was calculated from the equation

$$\gamma = \frac{[M] - [M_u]}{[M_m] - [M_u]} \qquad (5)$$

in which $[M_u]$ is the rotation of the undissociated substance, $[M_m]$ the rotation of the monion, and $[M]$ the observed rotation. From the values of γ (or directly from the rotation values) the dissociation constant was calculated by means of the relation.

$$pG' = pH - \log \frac{\gamma}{1 - \gamma} = pH - \log \frac{[M] - [M_u]}{[M_m] - [M]} \qquad (6)$$

It should be noted that from equation (5) either $[M_u]$ or $[M_m]$ can be calculated with $[M_m]$ or $[M_u]$ and a value of γ with the corresponding $[M]$ are known:

$$[M_m] = \frac{[M_u] (\gamma - 1) + [M]}{\gamma} \qquad (7)$$

Dextro-Alanine and Glycyl-Levo-Alanyl-Levo-Alanyl-Glycine.—For these ampholytes the two dissociation constants were calculated without making any assumptions in regard to the basic or acidic character of the two ionizing groups. The rotation of the molecular species existing in aqueous solution is designated as $[M_0]$, that on the acid side as $[M_1]$, and that on the alkaline side as $[M_2]$. Hence

$$\gamma_1 = \frac{[M] - [M_1]}{[M_0] - [M_1]} \qquad (8)$$

$$\gamma_2 = \frac{[M] - [M_0]}{[M_2] - [M_0]} \qquad (9)$$

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[18] Levene, P. A., Simms, H. S., and Bass, L. W., *J. Biol. Chem.*, 1926, lxx, 243.

Dextro-Alanyl-Dextro-Alanine Anhydride.—Preliminary experiments showed that this compound was hydrolyzed quite rapidly under the conditions of our experiments. It was therefore necessary to correct the data for hydrolysis, the correction being made as follows.

Levene and Pfaltz[14] showed that dextro-alanyl-dextro-alanine anhydride was rapidly hydrolyzed by a large excess of alkali and that under these conditions no perceptible racemization occurred.

A sample of the anhydride with 15 equivalents of alkali was allowed to stand at room temperature for 2 hours. Rotations of this hydrolyzed material were then taken for solutions containing 0, 1.000, 3.00, 5.00, and 15.00 equivalents of alkali, respectively. On the basis of the results (Table II) $\alpha_h = -0.19°$ was taken as the rotation in a 4.00 dm. tube of 0.0200 M solution of the hydrolyzed anhydride throughout the alkaline range.

The titration-rotation data were then determined. After each experiment samples of the solution were neutralized exactly and diluted to 0.0200 M. From the rotations of these solutions the degree of hydrolysis (h) was calculated by means of the relation

$$h = \frac{\alpha_0 - \alpha}{\alpha_0 - \alpha_h}, \tag{10}$$

where α_0 is the rotation of the anhydride in neutral solution (no hydrolysis), α_h the rotation of the hydrolyzed material (dipeptide), and α the rotation of the neutralized solution. Because of the low rotations the values of h (Column 7, Table III) were only approximate, but they were sufficiently accurate for the succeeding calculations.

The degree of dissociation of the enolized anhydride in each of the original solutions was then calculated by the following method. In each solution the observed rotation, calculated as molecular rotation [M_x], is the sum of two rotations:

$$[M_x] = h[M_h] + (1 - h) [M], \tag{11}$$

where h is the per cent of hydrolysis, [M_h] the molecular rotation of the hydrolyzed anhydride, and [M] the molecular rotation of the unhydrolyzed fraction (equilibrium mixture of the ionized and

[14] Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxiii, 661.

unionized substance). The true values for the rotations of the anhydride ([M]) were obtained from this equation (Column 8, Table III).

Since it was not possible to determine directly [M_m], the rotation of the completely ionized enolic form, its value was obtained by extrapolaton. The b' values, which are equal to γ, for 0.500, 0.800, and 1.000 equivalents of alkali were calculated from the titration data[15] (Column 3, Table III). The values for [M_m] calculated by equation (7) for these points were +72.5, +70.5, and +63.5, respectively. Using the average value +68.5 for [M_m], −46.5 for [M_u], and the rotations [M] of the anhydride at different degrees of dissociation, the values of γ (Column 9, Table III) were calculated from equation (5). The pG' values (Column 10, Table III) were calculated from equation (4).

4. Experimental Data.

Dextro-Alanine.—The dextro-alanine used in these experiments gave the following analysis.

No. 547.

    Calculated for $C_3H_7O_2N$. C 40.42, H 7.92, N 15.73.
    Found.   " 40.36, " 7.81, " 15.95 (Kjeldahl).

Individual samples of alanine equivalent to a 0.750 m solution were weighed into flasks. The solutions were then made up to 25.0 cc. with the required volumes of standard acid (or alkali) and water. The rotations were measured in 4.00 dm. tubes.

The data are recorded in Table I. The rotation values used for the calculation of γ_1 and γ_2 are [M_1] = 15.18, [M_0] = 2.13, and [M_2] = 4.80. Column 14 gives the deviations of pG' calculated on the basis of rotation from the average values determined by titration.

Dextro-Alanyl-Dextro-Alanine Anhydride.—The material used in these experiments gave the following analysis.

No. 656.

    Calculated for $C_6H_{10}O_2N_2$. C 50.69, H 7.09, N 19.72.
    Found.   " 50.32, " 7.26, " 19.79 (Kjeldahl).

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[15] The values of γ for higher concentrations of alkali were not reliable because of the hydrolysis which occurred.

TABLE I.

Dextro-Alanine. (0.750 Molar.)

| pH | $\frac{b-g}{c}$ | b' | Titration. | | | | α | $[M]$ | Rotation. | | | | Deviation of pG'. |
(1)	(2)	(3)	γ_1 (4)	γ_2 (5)	pG₁' (6)	pG₂' (7)	(8)	(9)	γ_1 (10)	γ_2 (11)	pG₁' (12)	pG₂' (13)	(14)
0.57	−1.300	−0.869					4.54	15.1_3					
0.63	−1.250	−0.861					4.57	15.2_4					
1.18	−1.000	−0.912	0.088		(2.20)		4.37	14.5_7	0.04_7		(2.48)		+0.09
1.93	−0.750	−0.735	0.265		2.37		3.51	11.7_0	0.26_8		2.37		−0.02
2.39	−0.500	−0.495	0.505		2.39		2.51	8.3_7	0.52_3		2.35		−0.04
2.39	−0.500	−0.495	0.505		2.39		2.54	8.4_4	0.51_4		2.37		−0.02
2.89	−0.250	−0.248	0.752		2.40		1.55	5.1_7	0.76_7		2.37		−0.02
5.83	0	0					0.63	2.1_0					
6.05	0	0					0.65	2.1_7					
9.32	0.250	0.250		0.250		9.80	0.78	2.6_0		(0.17_6)		(9.99)	+0.19
9.32	0.250	0.250		0.250		9.80	0.80	2.6_7		(0.20_2)		(9.91)	+0.11
9.79	0.500	0.500		0.500		9.79	1.02	3.4_0		0.47_6		9.83	+0.03
10.28	0.750	0.749		0.749		9.80	1.23	4.1_0		0.73_8		9.83	+0.03
11.63	1.000	0.989					1.43	4.7_7					
13.10	1.250	0.907					1.45	4.8_3					
Average..........					2.39	9.80							

A sample of anhydride equivalent to a 0.0500 M solution was weighed into a calibrated flask. 15 equivalents of alkali were added. The solution was diluted to 50.0 cc. and was then allowed to stand at room temperature for 2 hours. The rotation in a 4.00 dm. tube was −0.48°, which is equivalent to a rotation of −0.19° in a 4.00 dm. tube for a 0.0200 M solution.

Four 10.00 cc. samples of this mother solution were introduced into 25.0 cc. flasks. The calculated volumes of standard acid required to leave an excess of 0, 1.000, 3.00, and 5.00 equivalents of alkali respectively were added. The solutions were then diluted to 25.0 cc. and their rotations were measured. The experimental results are recorded in Table II.

TABLE II.

Rotation of Hydrolyzed Dextro-Alanyl-Dextro-Alanine Anhydride.

c	$\frac{b}{c}$	α
0.0200	0	−0.19
0.0200	1.000	−0.18
0.0200	3.00	−0.18
0.0200	5.00	−0.19
0.0500	15.00	−0.48
0.0200	15.00	−0.19

The titration-rotation data for dextro-alanyl-dextro-alanine anhydride were then determined by the following procedure. Individual samples equivalent to 0.0500 M solutions were weighed into 25.0 cc. flasks. The required volumes of standard alkali (or acid) were then added and the solutions were made up to volume. The rotations were measured as rapidly as possible and 10.00 cc. samples were taken at once for the measurement of the degree of hydrolysis by the method described in the following paragraph. The pH values of the original solutions were determined on the 15 cc. residues (Table III).

To determine the degree of hydrolysis in each of the above experiments, the 10.00 cc. samples of the original solutions were introduced into 25.0 cc. flasks. The quantities of standard acid (or

alkali) calculated for exact neutralization were then added and the solutions were made up to 25.0 cc.   The rotations are given in Column 6 of Table III.

Glycyl-Levo-Alanyl-Levo-Alanyl-Glycine.—The material used in these experiments gave the following analysis.

No. 624.
Calculated for $C_{10}H_{18}O_5N_4$.   C 43.76, H 6.64, N 20.44.
Found.   " 44.10, " 6.83, " 20.26 (Kjeldahl).

The following two series of experiments were run.

TABLE III.

Dextro-Alanyl-Dextro-Alanine Anhydride.   (0.0500 Molar.)

pH	$\frac{b-a}{c}$	b'	α	$[M_x]$	Neutralized solution.		Anhydride.		
					α	h	$[M]$ (correct-ed).	γ	pG'
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
						per cent			
0.78	−4.00		−0.91	−45.₅	−0.35		−45.₅		
7.05	0		−0.93	−46.₅	−0.37		−46.₅		
11.94	0.250		−0.88	−44.₀	−0.36		−44.₀	0.02₂	13.6
12.20	0.500	0.042	−0.83	−41.₅	−0.37		−41.₅	0.04₃	13.6
12.37	0.800	0.090	−0.72	−36.₀	−0.35		−36.₀	0.09₁	13.4
12.48	1.000	0.086	−0.74	−37.₀	−0.37		−37.₀	0.08₃	13.5
12.75	2.00		−0.57	−28.₅	−0.33	2₃	−30.₀	0.14₄	13.5
12.93	3.00		−0.47	−23.₅	−0.29	4₄	−23.₆	0.19₉	13.5
13.01	4.00		−0.34	−17.₀	−0.28	5₀	−10.₈	0.31₀	13.4
13.12	5.00		−0.27	−13.₅	−0.32	2₈	−9.₇	0.32₀	13.4
13.25	7.00		−0.15	−7.₅	−0.30	3₉	+2.₆	0.42₇	13.4
	15.00		+0.07	+3.₅	−0.36		+3.₅	0.43₅	
Average..									13.5

Series A.—A solution of 0.200 M tetrapeptide was prepared. Samples of 2.50 cc. plus the calculated volumes of standard acid (or alkali) were made up to 5.00 cc. with water.   The rotations were measured in 2.00 gm. tubes.   The results are recorded in Table V.

In the experiments above pH 7 the polariscope readings were difficult to make because of the formation of a small quantity of flocculent material upon the addition of standard alkali.

Series B.—Individual samples of the tetrapeptide equivalent to a 0.1000 M solution were weighed into flasks. The calculated volumes of standard acid (or alkali) were added and the solutions were made up to 25.0 cc. with water. These solutions were then centrifugalized to free them from the flocculent precipitate which obscured the readings in the experiments of Series A. The polariscope readings were then taken in 4.00 dm. tubes and the concentrations of the solutions were determined by analysis. The corrections for the concentrations are given in Table IV and the data of the titration-rotation experiments in Table V, together with the data from Series A.

TABLE IV.

Glycyl-Levo-Alanyl-Levo-Alanyl-Glycine.
Experiment B.

Correction of rotations to 0.1000 M concentration on the basis of total nitrogen determinations.

$\frac{b-a}{c}$ (1)	Total N in 1.00 cc. (2)	α (3)	α (corrected). (4)	[M] (5)	Amino N in 1.00 cc. (6)	$\frac{\text{Amino N}}{\text{Total N}}$. (7)
	mg.				mg.	
−0.500	5.39	13.54	13.94	348.5		
0 (Control.)	5.55				1.56	0.281
0.250	5.25	12.33	13.03	325.7		
0.750	5.46	12.18	12.37	309.2		
1.000	5.51	11.92	12.01	300.2	1.62	0.294
2.00	5.33	11.55	12.04	301.0	1.54	0.289
5.00	5.46	10.45	10.62	265.5	1.59	0.291

In some experiments of Series B, amino nitrogen determinations were made on the solutions. The constancy of the ratio of amino nitrogen to total nitrogen proved that no appreciable hydrolysis occurred, even in the solution containing 5 equivalents of alkali. The data are included in Table IV.

The values used in computing γ_1 and γ_2 from rotation data (Columns 11 and 12, Table V) are $[M_1] = 362.5$, $[M_0] = 331.0$, and $[M_2] = 300.6$. Column 15 of Table V gives the deviations of pG′ calculated on the basis of rotation data from the average values found by titration.

TABLE V.

Glycyl-Levo-Alanyl-Levo-Alanyl-Glycine. (0.1000 Molar.)

Experi- ment.	pH	$\frac{b-a}{c}$	b'	Titration.				Rotation.						Deviation of pG'.
				r_1	r_2	pG_1'	pG_2'	α	$[M]$	r_1	r_2	pG_1'	pG_2'	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
A	1.28	−1.500	−0.900	0.505				7.25	362.₈	0.45				
"	2.02	−1.000	−0.905	0.752				7.24	362.₀	0.79				
B	3.30	−0.500	−0.495			3.31		13.94	348.₈			3.39		+0.09
A	3.79	−0.250	−0.248			3.30		6.75	337.₈			3.22		−0.08
"	4.82	0	0					6.63	331.₈					
"	4.92	0	0					6.61	330.₈					
B	7.47	0.250	0.250		0.250		7.95	13.03	325.₇		0.17		8.16	+0.20
A	7.96	0.500	0.500		0.500		7.96	6.36	318.₀		0.43		8.08	+0.12
B	8.45	0.750	0.750		0.750		7.97	12.37	309.₂		0.72		8.04	+0.08
A	10.77	1.000	0.991					6.08	304.₀					
B	11.18	1.000	0.976					12.01	300.₂					
A	12.52	1.500	0.987					6.10	305.₀					
B	12.83	2.00	0.852					12.04	301.₀					
"	13.35	5.00	0.831					10.62	265.₈					
Average.........						3.30	7.96							

ON WALDEN INVERSION.

IX. ON THE MECHANISM OF HYDROLYSIS OF OPTICALLY ACTIVE PROPYLENE OXIDE.

BY P. A. LEVENE AND A. WALTI.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In order to understand fully the mechanism of the hydrolysis of propylene oxide, the configurational relationship of propylene oxide to propylene glycol must be established. Dextro-propylene oxide may be prepared from levo-1-chloro-2-hydroxypropane (I) or from dextro-1-hydroxy-2-chloropropane (III).

$$
\begin{array}{ccccc}
CH_2Cl & & CH_3 & & CH_2OH \\
| & & | \diagdown & & | \\
HCOH & \longrightarrow & HC \quad {>}O & \longleftarrow & HCCl \\
| & & | \diagup & & | \\
CH_3 & & CH_3 & & CH_3 \\
\text{Levo.} & & \text{Dextro.} & & \text{Dextro.} \\
\text{I.} & & \text{II.} & & \text{III.}
\end{array}
$$
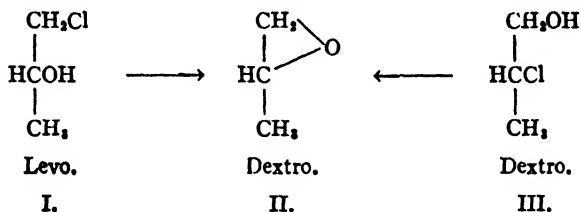
In these two transformations it is reasonable to assume that the first proceeds without inversion, inasmuch as reactions of substitution of the hydrogen on a hydroxyl group were shown by Kenyon and Phillips[1] and their coworkers to proceed normally, whereas substitution of a hydroxyl may bring about inversion.

In the present case the assumption may be substantiated by the following argument. From the work of Levene and Haller[2] and Levene and Walti[2] it follows that levo-1-chloro-2-hydroxy-propane has the configuration of levo-lactic acid; that is, it belongs to the *d*

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[1] Kenyon, J., Phillips, H., and Turley, H., *J. Chem. Soc.*, 1925, cxxvii, 399.

[2] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxv, 49; 1926, lxvii, 329. Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1926, lxviii, 415.

series and hence has the hydroxyl to the right.   Hudson[3] has demonstrated for a number of lactones of hydroxy acids that the direction of their rotation is determined by the allocation of the hydroxyl, those having the latter on the same side as levo-lactic acid (d) rotating to the right.   On the basis of this rule, the configuration of levo-lactic acid (d) may be assigned to dextrorotatory propylene glycol. Abderhalden and Eichwald[4] held the opposite and erroneous view.

The question arises as to the reaction from (III) to (II).   Abderhalden and Eichwald[4] have shown that dextro-1-hydroxy-2-chloropropane is oxidized to levo-chloropropionic acid.   This acid, on the basis of considerations of Clough,[5] of Kenyon, Phillips, and Turley,[1] and of Levene and Mikeska,[6] is configurationally related to levo-lactic acid (d); therefore the conclusion is justified that levo-1-chloro-2-hydroxypropane (I) and dextro-1-hydroxy-2-chloropropane (III) are configurationally related; and hence a second conclusion follows, that both reactions (one from (I) to (II) and the other from (III) to (II)) proceed without inversion.   The significance of this observation will be referred to later.   Thus, the reaction of formation of propylene oxide proceeds normally, the reverse reaction of hydrolysis either normally or abnormally depending upon the external conditions.

Abderhalden and Eichwald observed that the product obtained on hydrolysis of dextro-propylene oxide was at times dextrorotatory, at times levorotatory.   As a rule, under the conditions employed by them, it was dextrorotatory.   They justly remarked that the variations in direction of rotation could be explained either on the assumption of formation of some intermediate substances or on the assumption of Walden inversion in one case.   These authors realized that the problem required further inquiry.   Nevertheless, they assumed configurational relationship between levo-1-chloro-2-hydroxypropane and dextro-propylene glycol on the basis of the fact that the latter was formed from dextro-1-hydroxy-2-chloropropane and that it

[3] Hudson, C. S., J. Am. Chem. Soc., 1910, xxxii, 338.

[4] Abderhalden, E., and Eichwald, E., Ber. chem. Ges., 1918, li, 1312.

[5] Clough, G. W., J. Chem. Soc., 1918, cxiii, 526.

[6] Levene, P. A., and Mikeska, L. A., J. Biol. Chem., 1926, lxx, 365.

could also be transformed into levo-1-bromo-2-hydroxypropane. Their assumption was erroneous.

The progress of hydrolysis of dextro-propylene oxide has now been studied in some detail. It was found that the direction of rotation of the resulting glycol depends on the hydrogen ion concentration. A comparatively high hydrogen ion concentration of the hydrolysis medium leads from a dextrorotatory oxide to a dextrorotatory glycol. On the other hand, a high hydroxyl ion concentration leads to a levorotatory glycol. Inasmuch as from the previous work of Levene and Walti it is known that levo-1-chloro-2-hydroxypropane is configurationally related to levo-propylene glycol and hence that levorotatory propylene glycol is configurationally related to dextrorotatory propylene oxide, it now follows that hydrolysis with alkalies proceeds normally without inversion and that hydrolysis with acids proceeds with inversion.
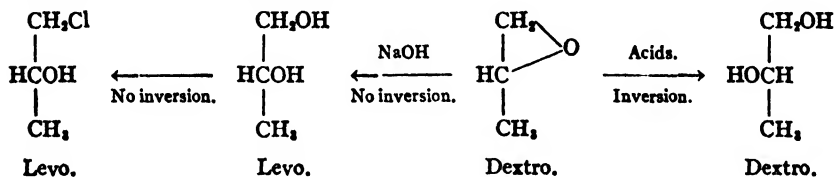
In Table I are given the results of hydrolysis under the influence of various catalysts. It must be mentioned here that from the rotation of the reaction product alone, one is not justified in drawing conclusions regarding the direction of rotation of the glycol. The reaction product contains, in addition to the latter, condensation products of higher molecular weight, esters, etc. The conclusions formulated above were all based on experiments in which the glycol had been isolated.

Discussion of the Theories of Walden Inversion Occurring during Hydrolysis.

The type of Walden inversion described here has been observed in recent years on several occasions and every observer has emphasized the importance of it, inasmuch as it brings to our attention the possibility that every hydrolysis of an ester having one or both components optically active may lead to a change in the configuration

of the active components.   The same occurrence may take place on hydrolysis of lactones of optically active hydroxy acids.

Hence, it is important to be able to recognize those conditions of hydrolysis or of alcoholysis which lead to an inversion.   The problem having been formulated only recently, there is at present comparatively little experimental material to permit comprehensive conclusions.

The two observations which are nearest in their character to the one described here are the one by Holmberg on propiolactone-β-carboxylic acid (malic acid β-lactone) and the other by Richard Kuhn and coworkers on ethylene oxide dicarboxylic acid.

Holmberg[7] assumes that dextro-malic acid β-lactone is configurationally related to dextro-malic acid.   Hydrolysis with alkalies leads to the dextro acid.   Hence, according to Holmberg, the reaction proceeds without inversion.   On the other hand, acid hydrolysis leads to a levo acid; hence, in the opinion of Holmberg, acid hydrolysis brings about an inversion.

If the assumptions of Holmberg are correct, then the conclusions of this author in regard to dextro-malic acid β-lactone coincide with ours in regard to propylene oxide and a general rule might be formulated connecting Walden inversion with cation hydrolysis and the normal reaction with anion hydrolysis.   Such a hypothesis was in fact suggested by Gadamar[8] as a general rule for Walden inversion.

In reality this comprehensive assumption is contradicted by recent observations of Kuhn and coworkers who found that in ethylene oxide dicarboxylic acid both the cis and the transforms are hydrolyzed in the same sense by either acids or alkalies.

Kuhn and Ebel[9] then offered the following explanation for the observations of Holmberg on dextro-malic acid β-lactone.

A   B

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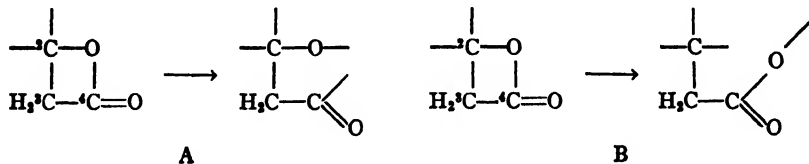
[7] Holmberg, B., *J. prakt. Chem.*, 1913, lxxxvii, 456, 553.

[8] Gadamar, J., *J. prakt. Chem.*, 1913, lxxxvii, 312.

[9] Kuhn, R., and Ebel, F., *Ber. chem. Ges.*, 1925, lviii, 919.

This explanation states that in reaction A (without inversion) the asymmetric carbon atom remains saturated all through the reaction and that in reaction B at some phase of the process the asymmetric carbon atom approaches a trivalent state.

Similar assumptions were recently made by Phillips[10] and by Kenyon, Phillips, and Turley.[1] One of the important transformations discovered by these investigators is the following:

$$C_6H_5CH_2\diagup\overset{OEt}{\underset{CH_3\diagup\,^\diagdown H}{C}} \quad \xleftarrow{\text{EtBr}} \quad C_6H_5CH_2\diagup\overset{O\!:\!K}{\underset{CH_3\diagup\,^\diagdown H}{C}} \quad \xrightarrow{\text{Et}\,OSO_2C_7H_7}$$

Dextro.   Dextro.

$$C_6H_5CH_2\diagup\overset{OEt}{\underset{CH_3\diagup\,^\diagdown H}{C}}$$

Dextro (no inversion).

$$C_6H_5CH_2\diagup\overset{OSO_2C_7H_7}{\underset{CH_3\diagup\,^\diagdown H}{C}} + C_6H_5O\!:\!K = C_6H_5CH_2\diagup\overset{H}{\underset{CH_3\diagup\,^\diagdown OC_6H_5}{C}} + KOSO_2C_7H_7.$$

Dextro.   Levo (inversion).
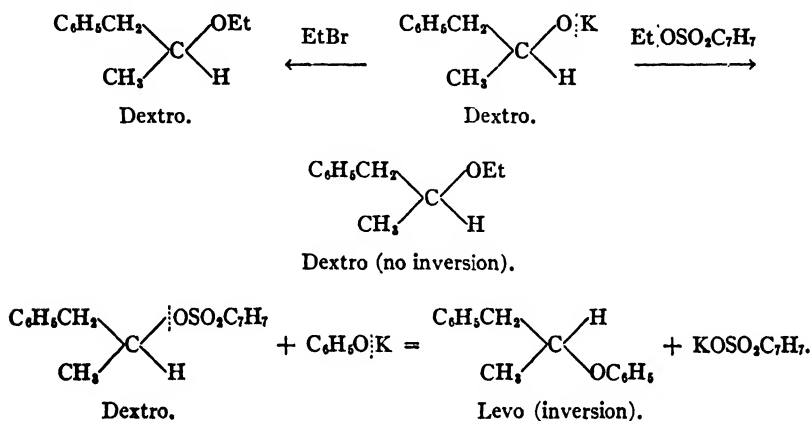
The dotted lines indicate the place of substitution.

The explanations of Kuhn and of Phillips and of Kenyon and associates may be taken to signify that Walden inversion occurs when the substitution takes place directly on the asymmetric carbon atom. This assumption is in conformity with all the ideas on Walden inversion. It expresses a necessary condition for inversion; however, in itself it is not a sufficient condition inasmuch as otherwise all reactions of substitution on an asymmetric carbon atom should lead to a Walden inversion.

In all theories of Walden inversion the emphasis has been laid on the place on the surface of the asymmetric carbon atom which permitted the entrance of the substituting group. As the groups attached to the asymmetric carbon atom shift through the action of the reagent, room is created for the entering radicle. The position of this free entering space (or the "unsaturated area" as termed by

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[10] Phillips, H., *J. Chem. Soc.*, 1925, cxxvii, 2552.

some investigators) determines the occurrence or non-occurrence of a Walden inversion.

In all discussions on Walden inversion it seems to us that too little attention was devoted to the possible interreaction of the groups of lesser polarity.   The substitution or principal reaction takes place between the most polar groups of the reacting substances.   For convenience of discussion these may be referred to as "groups of the first order" and the remaining atoms or radicles as "groups of the second order."

Assuming that the first step in any reaction of substitution is the formation of a complex in the sense of Kekulé, one may further assume that in this complex the tetrahedron of the asymmetric carbon atom possesses a different shape from that in the original substance and hence that the distances between the groups of the second order will be changed, thus enhancing or lowering the chance of their interreacting.   The greater the original asymmetry of the tetrahedron, the greater will be the change produced in the complex.

With the background of these considerations, the outcome of a reaction of substitution may be viewed as the result of two reactions: one involving the two highly polar groups (groups of the first order) and the other involving the groups of lesser polarity (groups of the second order).   When the velocity of the first reaction is much greater than that of the second, no Walden inversion will occur.   When that of the second is greater, either inversion or racemization or both reactions may take place.

The advantage of this assumption lies in the fact that it permits an experimental investigation of the respective velocities: the first may be measured directly and the second may be studied by means of the velocities of racemization.

The outlined suggestion is in part the outcome of observations on Walden inversion in the series of secondary alcohols made in this laboratory by Levene and Mikeska which will be discussed later.

EXPERIMENTAL.

Optically Active Propylene Oxide.—Optically active propylene oxide was prepared according to our earlier publications.   It may be mentioned that in one case the propylene oxide was prepared without

TABLE I.

Experiment No.	Amount of acid or alkali.	Amount of water.	Amount of propylene oxide.	Temperature.	Length of time.	Rotations in 1 dm. tube.
		cc.	cc.	°C.	hrs.	degrees
1	0.0800 gm. d-tartaric acid.	7.5	4.5 (α = +9.55°)	64–65	16	+2.30
2	0.10 cc. formic acid.	2.0	1.2 "	64–65	16	+1.57
3	2.5 " N H₂SO₄.		1.0 "	46	17	+1.16
4	2.5 " N₃PO₄.		1.0 "	46	17	+1.28
5	0.034 gm. quinic acid.	2.5	1.0 "	46	17	+1.00
6	0.030 " camphor-sulfonic acid.	2.5	1.0 "	64–65	17	+1.54
7	0.042 " oxalic acid.	2.5	1.0 "	64–65	17	+1.34
8	0.1659 " "	5	2.0 (α = +8.51°)	33–34	16	+1.13
9	0.3318 " "	5	2.0 "	33–34	16	+1.25
10	0.6626 " "	5	2.0 "	33–34	16	+1.25
11	0.220 " "	5	5.0 "	33–34	16	+1.84
12	10 cc. N HCl.		3.2 "	50	18	−0.32*
13	2.5 " 2 N HCl.		1.0 (α = +10.50°)	65–80	17	−0.01*
14	2.6 " 4 " "		1.0 "	65–80	17	−0.80*
15	2.5 " N NaOH.		1.0 "	64–65	17	−7.50
16	4 " " "		2.0 "	35–36	15	−8.85
17	5 " " "		2.5 "	36–37	18	−8.02

* The small levorotation in these experiments was due to the presence of small quantities of levo-chlorohydrin, inasmuch as the glycol isolated was dextrorotatory.

a prior distillation of the crude propylene bromohydrin.   Thus, 52 gm. of crude propylene bromohydrin (residue of the chloroform extract obtained on treatment of 40 gm. of propylene glycol ($[\alpha]_D = -13.71°$)) with 23 gm. of potassium hydroxide and 30 cc. of water gave 18 gm. of propylene oxide ($[\alpha]_D = +8.51°$).

Hydrolysis of Optically Active Propylene Oxide.—All experiments on the hydrolysis of propylene oxide were carried out in sealed tubes. The conditions employed in each experiment are recorded in Table I. After cooling to room temperature the rotations of the aqueous solutions were taken in a 1 dm. tube.

Dextro-Propylene Glycol from Dextro-Propylene Oxide by Means of Tartaric Acid.—From Experiment 1 the dextro-propylene glycol was isolated by evaporating the aqueous solution under reduced pressure.   The residue was taken up in alcohol and the solution was concentrated under reduced pressure until all water and alcohol were removed; the glycol was then distilled at 92°C. and 15 mm. It showed a rotation of $\alpha = +4.11°$ without solvent.   In order to confirm this result 1 gm. of the propylene glycol obtained in this manner was transformed into the di-(phenylurethane) by adding 3.3 gm. of phenylisocyanate and heating at 100–115°C. for 1 hour.   The urethane was isolated as previously described.   It had a melting point of 145–146°C. and analyzed as follows:

0.1000 gm. substance required 6.02 cc. 0.1 N HCl.
$$C_{17}H_{18}N_2O_4. \quad \text{Calculated.} \quad N \ 8.91.$$
$$\text{Found.} \qquad " \ 9.03.$$

The rotation of the substance in absolute alcohol was as follows:

$$[\alpha]_D^{n} = \frac{-0.19° \times 100}{1 \times 4} = -4.75°.$$

Dextro-Propylene from Dextro-Propylene Oxide by Means of Oxalic Acid.—From the reaction products of Experiments 8, 10, and 11, dextro-propylene glycol was isolated in the manner described above.

In Experiment 8 the isolated propylene glycol had the following rotation in water.

$$[\alpha]_D^{20} = \frac{+0.52° \times 100}{1 \times 17.2} = +3.02°.$$

In Experiment 10 the propylene glycol had the following rotation in water.

$$[\alpha]_D^{20} = \frac{+ 0.51° \times 100}{1 \times 16.4} = + 3.10°.$$

In each of these two experiments the yield of propylene glycol was 1.3 gm.

In Experiment 11 the rotation of the propylene glycol in water was as follows:

$$[\alpha]_D^{20} = \frac{+ 0.61° \times 100}{1 \times 16.9} = + 3.60°.$$

The rotation of the propylene glycol without solvent was $\alpha_D = +3.25°$. In the latter series of experiments it was observed that a neutral, water-insoluble, ether-soluble compound was formed in small quantities. This compound probably represents a neutral ester of oxalic acid and propylene glycol.

Dextro-Propylene Glycol from Dextro-Propylene Oxide by Means of Hydrochloric Acid.—In Experiments 12, 13, and 14 with hydrochloric acid, the reaction mixture was levorotatory. As this reaction mixture was neutral toward litmus and did not contain free chlorine ions, the hydrochloric acid was apparently bound to the oxide. Thus, dextro-propylene oxide forms levopropylene chlorohydrin. In order to isolate the propylene glycol the calculated amount of 2 N hydrochloric acid was slowly added in the cold to 3 cc. of dextro-propylene oxide ($\alpha = +9.5°$). The rotation of the solution was $\alpha = -0.71°$ in a 1 dm. tube. The solution was concentrated under diminished pressure and the viscous residue made up to 2.5 cc. with water. The rotation was $\alpha = +2.03°$. The solution was again concentrated until all water was removed and from the residue the urethane of propylene glycol was prepared. It melted at 145–146°C. and analyzed as follows:

0.0500 gm. substance required 3.17 cc. 0.1 N HCl.
$C_{17}H_{16}N_2O_4$.   Calculated.   N 8.91.
                    Found.   " 8.88.

Alkaline Hydrolysis of Optically Active Propylene Oxide.—The optically active propylene oxide in Experiment 16 was hydrolyzed

with normal sodium hydroxide solution. After the polarimetric reading had been taken the solution was made neutral to litmus with hydrochloric acid and made up to 10 cc. with water. The rotation was $\alpha = -5.15°$. The solution was evaporated under diminished pressure; the residue was taken up in absolute alcohol, freed from the latter, and the propylene glycol was then distilled at 92° and 15 mm.; the yield was about 0.8 gm. The rotation was as follows:

$$[\alpha]_D^{20} = \frac{-1.55° \times 100}{1 \times 6.20} = -25.0°.$$

After the distillation of the propylene glycol, a residue was left which gave the following rotation in water.

$$[\alpha]_D^{20} = \frac{-3.53° \times 100}{1 \times 9.34} = -37.8°.$$

A certain amount of this product in the propylene glycol just described caused its higher rotation.

In order to find the nature of this residue, Experiment 17 was started and worked up as stated above. The propylene glycol obtained from this experiment had a rotation in water of

$$[\alpha]_D^{20} = \frac{-1.52° \times 100}{1 \times 6.74} = -22.55°.$$

To 0.25 gm. of this propylene glycol was added 0.8 gm. of phenylisocyanate and the mixture was heated for 1 hour at 100–105°. After cooling, the solid product was washed in a mortar with very little ether, filtered under suction, and crystallized from dilute alcohol. The melting point was 145–146°C. and the substance analyzed as follows:

0.0110 gm. substance: 0.962 mg. N.
$C_{17}H_{18}N_2O_4$.   Calculated.   N 8.91.
                      Found.   " 8.75.

The rotation of the di-(phenylurethane) in absolute alcohol was

$$[\alpha]_D^{20} = \frac{+0.43° \times 100}{1 \times 3.60} = +11.94°.$$

The substance which distilled (after the propylene glycol) at 105–110°C. and 15 mm. probably represents

```
CH₃        CH₃            CH₃        CH₃           CH₃
 |          |              |          |             |
CHOH       CHOH    or    CH—O—CH    or    CH—O—CH₂
 |          |              |          |             |
CH₂—O—CH₂              CH₂OH    CH₂OH        CH₂OH    CHOH
                                                       |
                                                      CH₃
```
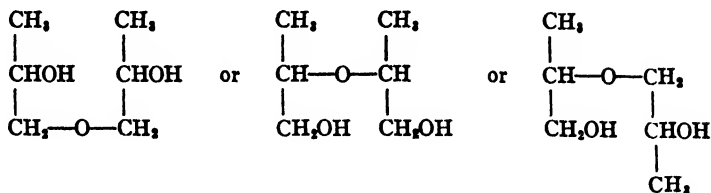
0.0936 gm. substance: 0.1808 gm. CO_2 and 0.0886 gm. H_2O.
$C_6H_{14}O_3$. Calculated. C 53.73, H 10.45.
Found.   " 52.67, " 10.69.

The rotation of the substance in water was

$$[\alpha]_D^{20} = \frac{-4.38° \times 100}{1 \times 11.34} = -38.62°.$$

Preparation of Dextro-Propylene Glycol of Higher Optical Activity. —5 cc. of dextro-propylene oxide ($\alpha = +8.51$) were added to 8 cc. of anhydrous formic acid and the glycol was isolated according to the directions of Abderhalden and Eichwald.   3 gm. of dextro-propylene glycol were obtained which had a rotation in water of

$$[\alpha]_D^{20} = \frac{+1.78° \times 100}{1 \times 15.9} = +11.2°.$$

The rotation of the glycol without solvent was about +8°.[11]

This dextro-propylene glycol was transformed into dextro-propylene bromohydrin by the methods which have been described previously.   The rotations of the dextro- and of the levo-propylene bromohydrin were taken in benzene and chloroform.

The rotations of the dextro-propylene bromohydrin were as follows:

1. In chloroform solution $[\alpha]_D^{20} = \frac{+0.77° \times 100}{1 \times 14.2} = +5.42°.$

2. In benzene solution $[\alpha]_D^{20} = \frac{+0.59° \times 100}{1 \times 14.8} = +3.99°.$

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[11] On account of layer formation the rotation could not be taken very accurately.

Levo-propylene bromohydrin was prepared by the addition of hydrobromic acid to dextro-propylene oxide. To 14 gm. of propylene oxide ($\alpha = +10.44°$) was added slowly under cooling a solution of 46 gm. of hydrobromic acid (sp. gr. 1.45) in 60 cc. of water. The bromohydrin was taken up in chloroform, neutralized with sodium carbonate, dried with sodium sulfate, and distilled under diminished pressure. 10 gm. of levo-propylene bromohydrin were obtained.

The substance analyzed as follows:

0.1221 gm. substance: 0.1642 gm. AgBr.

C_3H_7OBr. Calculated. Br 57.55.

Found.   " 57.23.

The rotations of the levo-propylene bromohydrin were:

1. In chloroform solution $[\alpha]_D^{20} = \dfrac{-1.62° \times 100}{1 \times 20.0} = -8.10°$.

2. In benzene solution $[\alpha]_D^{20} = \dfrac{-1.22° \times 100}{1 \times 20.9} = -5.83°$.

3. Without solvent $[\alpha]_D^{20} = \dfrac{-2.32°}{1 \times 1.599} = -1.45°$.

AU SUJET DE LA TECHNIQUE DE LA CULTURE DES TISSUS.

Par ALEXIS CARREL.

(*Des laboratoires, The Rockefeller Institute for Medical Research.*)

Il y a quatre ans, j'ai décrit une technique (1) qui permet d'obtenir la croissance continue de colonies cellulaires dans des flacons où elles se développent sous l'influence d'un milieu composé de deux parties, l'une solide et l'autre liquide. La partie solide est constituée par un coagulum de fibrine qu'on lave de temps en temps avec de la solution de Locke ou de Tyrode. La partie liquide, placée à la surface du coagulum, contient les substances nutritives et peut être renouvelée aussi souvent qu'il est utile. Pendant ces dernières années, la méthode a été employée dans un très grand nombre d'expériences. Nous avons donc pu l'améliorer de façon considérable. Ce sont ces perfectionnements qui font l'objet de la présente note.

1° Les flacons plats à col oblique dans lesquels croissent les tissus, appartenaient à plusieurs types, dont les uns étaient destinés à l'étude physiologique des tissus, et les autres permettaient aussi l'étude morphologique des cellules. L'expérience a montré que le plus commode de tous ces flacons est le type D de 5 cm. de diamètre, qui ne présente pas d'autre ouverture que celle du col oblique. Les tissus peuvent y être cultivés dans 2 c.c. de milieu. Le flacon du même type qui a 8 cm. de diamètre est inutilement grand. Le type à deux cols est moins commode et n'est plus employé que dans les expériences où l'on étudie l'effet d'un gaz sur la croissance des tissus. On ne se sert plus des flacons des autres types que de façon exceptionelle. Les flacons à fond mobile qui servaient à l'étude morphologique des tissus ont été complètement abandonnés. Ils seront remplacés par des boîtes de verre taillé, dont l'étude se termine en ce moment, et qui permettent l'emploi de l'objectif à immersion. Il est important que ces flacons soient en verre de bonne qualité et

(1) A. Carrel. *Journ. of exper. Med.*, 1923, t. XXXVIII, p. 407. *C. R. de la Soc. de biol.*, 1923, t. LXXXIX, p. 1017.

que leur forme soit régulière. Le fond surtout doit être plat, afin que l'épaisseur du milieu soit uniforme. S'il est bosselé, la croissance des colonies se fait de façon irrégulière. Le verre doit être assez transparent pour permettre l'examen microscopique à un grossissement de 125 diamètres environ. Les modifications des flacons qui ont été préconisées par quelques expérimentateurs ne constituent pas, jusqu'à présent, un progrès. En particulier, l'emploi du flacon à tube horizontal de Borrel enlève à la méthode presque toute sa valeur. La fermeture des flacons se fait, à la fois, par un tampon de coton non absorbant et par un capuchon de caoutchouc qui adhère exactement au goulot afin d'empêcher toute évaporation. Le tampon de coton est placé, avant la stérilisation, dans le fond du capuchon, de telle sorte qu'il s'applique de lui-même sur l'ouverture du flacon.

2° Les tissus sont toujours placés dans un coagulum de plasma qui leur sert de support. Les sangs de Poule, de Cobaye, de Rat, de Lapin, de Chien et d'Homme peuvent être également employés. Cependant, le sang de Poule donne les caillots le plus solides. On injecte d'abord le plasma, en général, 0,5 c.c. Puis on secoue le flacon afin que le liquide s'étende sur la surface du verre. Le caillot est ainsi plus adhérent. On ajoute ensuite 1 c.c. de solution de Tyrode, et 0,5 c.c. d'extrait dilué de pulpe d'embryon. Les fragments de colonies cellulaires sont introduit avec une spatule ou une pipette. Au bout de quelques minutes, la coagulation se produit. Suivant la nature de l'expérience, on lave le caillot plus ou moins longuement. Puis on place à sa surface 1 ou 2 c.c. du liquide dont on veut étudier l'effet sur les cellules. Le volume du milieu solide est suffisant pour permettre le développement du tissu, surtous si le fond du flacon est bien plat. Comme, dans la plupart des cas, il faut retirer le caillot du flacon au bout de quelques jours ou de quelques semaines, il est utile de le renforcer. En effet, si on essaye de décoller de la surface du verre le coagulum tel qu'il est obtenu par la technique décrite plus haut, il se déchire et les tissus sont détruits. Il faut le renforcer préalablement à l'aide de 0,5 c.c. de plasma et 0,5 c.c. d'extrait dilué. On obtient ainsi un coagulum qui, au bout de 8 ou 10 jours, possède une élasticité et une consistance telles qu'on peut facilement le retirer du flacon à l'aide d'une spatule de platine.

3° Cette petite opération se fait chaque fois qu'on désire examiner

les tissus à un fort grossissement, ou bien quand il s'agit de les trans-
férer dans un milieu frais.   Il est inutile de se servir d'instruments
compliqués.   Unes patule flexible de platine suffit.   On commence
par détacher le caillot dans le voisinage du col, la spatule étant droite.
Puis, par pression sur les parois du col, on donne à la tige de la spatule
la courbure convenable pour détacher le caillot de la circonférence
du flacon et de sa surface.   Finalement, on verse le caillot sur une
plaque de verre stérilisée où il est débité en fragments rectangulaires
à l'aide d'un couteau à cataracte.   Ces fragments sont lavés, puis
placés dans d'autres flacons, ou bien fixés sur des lamelles de verre,
pour l'examen avec l'objectif à immersion.

4° L'expérience acquise au cours de milliers d'expériences nous a
montré que le lavage des cultures doit être plus prolongé et fait avec
une quantité plus grande de liquide que nous ne le pensions au début.
Il faut employer au moins 3 c.c. de solution de Tyrode pendant 15
minutes.   Dans beaucoup d'expériences, on lave le coagulum dans
6 c.c. de solution de Tyrode pendant 30 minutes.   Ce lavage se fait
tous les 2, 3 ou 4 jours, suivant la nature du milieu liquide.   Malgré
la fréquence des manipulations, les risques d'infection bactérienne
sont extrêmement petits.   Le nombre des flacons infectés est très
inférieur à 1 p. 100.   L'importance de cette complication est devenue
négligeable.

Grâce à cette technique, la cultures des tissus est devenue d'un
emploi beaucoup plus facile.   On peut s'en servir aujourd'hui avec
le plus grand profit dans l'étude de presque tous les problèmes im-
portants de la physiologie et de la pathologie.

LES MILIEUX NUTRITIFS ET LEUR MODE D'EMPLOI DANS LA CULTURE DES TISSUS.

Par ALEXIS CARREL.

(*Des laboratoires, The Rockefeller Institute for Medical Research.*)

Nous savons depuis longtemps que les fibroblastes et les cellules épithéliales ne se multiplient pas dans un milieu composé de sérum sanguin, tandis que les monocytes du sang, les macrophages des tissus et les macrophages sarcomateux y prolifèrent abondamment (1*). Il est bien connu aussi que les cellules épithéliales et conjonctives demandent, pour la synthèse de leur protoplasma, certaines protéines contenues dans le suc des tissus embryonnaires (2*). C'est grâce à ces sucs qu'une race de fibroblastes a pu se propager depuis quinze ans avec une rapidité telle que chaque colonie double de volume en 48 heures. D'autres substances nutritives ont été découvertes récemment. Les produits de l'hydrolyse incomplète de certaines protéines déterminent la multiplication des fibroblastes et des cellules épithéliales avec une vélocité parfois plus grande que les sucs embryonnaires (3*). Au cours du très grand nombre d'expériences faites pendant ces dernières années, la composition et le mode d'emploi des différents milieux ont été modifiés de façon considérable. Ce sont ces progrès d'ordre pratique qui font l'objet de cette communication.

1° Le coagulum de fibrine est un des éléments essentiels du milieu de culture. L'expérience a démontré que l'étude physiologique des tissus est impossible quand on se sert seulement d'une lame de verre,

(1*) A. Carrel et A. H. Ebeling. *Journ. of exper. Med.*, 1922, t. XXXVI, p. 365; 1923, t. XXXVII, p. 759; t. XXXVIII, p. 487, 513. *C. R. de la Soc. de biol.*, 1923, t. LXXXIX, p. 1144, 1261.

(2*) A. Carrel. *Journ. of exper. Med.*, 1913, t. XVII, p. 14. L. E. Baker et A. Carrel. *C. R. de la Soc. de biol.*, 1926, t. XCV, p. 157. *Journ. of exper. Med.*, 1926, t. XLIV, 387.

(3*) A. Carrel. *C. R. de la Soc. de biol.*, 1926, t. XCIV, p. 1060. A. Carrel et L. E. Baker. *C. R. de la Soc. de biol.*, 1926, t. XCV, p. 359. *Journ. of exper. Med.*, 1926, t. XLIV, p. 503.

comme le fait Lewis, d'un réseau de toile d'araignée, à la manière
de Harrison, ou d'agar, suivant la pratique de plusieurs auteurs.   Ces
procédés sont d'une application très limitée et leur valeur négligeable
dans l'étude de la biologie des tissus.   Jusqu'à présent, le coagulum
de fibrine est le meilleur support que nous possédions pour la migra-
tion et la multiplication des cellules.   Le plasma des différents
animaux de laboratoire produit des caillots qui varient en trans-
parence, adhérence, rétractilité, friabilité, etc.   Les coagulums les
plus favorables sont ceux qui ne se contractent pas et permettent aux
cellules de se multiplier et d'émigrer dans le réseau defibrine pendant
plusieurs semaines.   Le sang de Poule fournit le meilleur milieu.
Les fibroblastes peuvent s'y développer de façon continue pendant
10 à 15 jours, et les macrophages pendant 30 ou 50 jours.   Le caillot,
friable au début, devient peu à peu élastique.   On peut le retirer du
flacon sans qu'il se contracte et perde son sérum, et en extirper les
colonies cellulaires qu'il contient.   Le plasma de Cobaye est aussi
d'une consistance et d'une transparence convenables.   Le coagulum
du plasma humain se liquéfie avec une grande facilité.   Celui du
Lapin devient rapidement opaque, et se contracte souvent en une
lame mince en perdant son sérum.   Celui du Rat présente des in-
convénients analogues, quoique à un moindre degré.   Ce sont donc
les coagulums du sang de Poule et de Cobaye qui sont les plus favora-
bles, et que nous employons le plus souvent.   Comme la présence du
sérum est, dans certaines expériences, un inconvénient sérieux, nous
avons, il y a plusieurs années, établi une technique permettant
d'utiliser non pas le plasma sanguin, mais une solution de fibrino-
gène (4*).   On peut obtenir ainsi un caillot excellent, quoique moins
solide et durable que celui provenant du plasma.   Cependant, nous
avons presque complètement abandonné l'emploi du fibrinogène pour
le procédé suivant qui est plus simple.   La culture est préparée avec
du plasma.   Mais, après coagulation, on se débarrassee du sérum par
le lavage du caillot.   Si on lave la culture à trois reprises, pendant
15 minutes, dans 6 c.c. de solution de Tyrode, la plus grande partie
du sérum est éliminée.   Ce qui en reste se trouve sous une concen-
tration trop faible pour exercer un effet appréciable sur les tissus.
Dans beaucoup d'expériences, un seul lavage d'une durée de 30

(4*) A. H. Ebeling. *Journ. of exper. Med.*, 1921, t. XXXIII, p. 641.

minutes suffit. Le caillot ainsi obtenu est très solide. Il faut néanmoins surveiller chaque jour son état et le renforcer de temps en temps dans le points où il se raréfie, surtout quand il s'agit de culture d'épithélium, ou de cellules malignes.

2° Le milieu nutritif des monocytes et des macrophages se compose, d'habitude, de sérum sanguin. Il suffit d'ajouter un tiers ou un sixième de sérum au milieu total pour obtenir une multiplication abondante des cellules. Le liquide peut rester de 3 à 4 jours sans être changé. Nous avons récemment substitué au sérum le plasma hépariné. On sait que l'héparine, découverte par Howell (5*), permet au plasma de rester liquide indéfiniment dans un tube paraffiné, ou parfaitement propre, quand sa concentration est de 1 p. 10.000. Si le coagulum est soigneusement lavé, on peut le couvrir de plasma hépariné, sans que celui-ci se coagule. Il devient ainsi possible d'étudier la multiplication des cellules non plus dans du sérum qui est un produit artificiel et parfois toxique, mais dans du plasma vrai, qui constitue un milieu beaucoup plus favorable que le sérum. Mais, pas plus que le sérum, le plasma n'a la propriété de déterminer la multiplication des cellules épithéliales et des fibroblastes. Le plasma hépariné est, ainsi que les suspensions de fragments musculaires, et les précipités de protéines, un excellent milieu pour les macrophages.

3° Le suc d'embryon de Poule est devenu d'un emploi beaucoup plus général depuis que nous avons trouvé que son effet n'est pas spécifique (6*). Des tissus de Lapin, de Rat, ou d'Homme croissent aussi bien dans du suc d'embryon de Poule que dans le suc des tissus embryonnaires homologues. La pulpe, produite par l'appareil de Latapie, est diluée dans 3 fois son volume de solution de Tyrode, centrifugée, congelée pendant 15 minutes à—50°, et centrifugée de nouveau. On obtient ainsi un liquide clair, qui ne contient aucune cellule vivante, et dont les protéines sont utilisées par la plupart des cellules fixes. La découverte du rôle des peptones et des protéoses nous a permis de

(5*) W. H. Howell et E. Holt. *Amer. Journ. of Physiol.*, 1918, t. XLVII, p. 328. W. H. Howell. *Amer. Journ. of Physiol.*, 1922, t. LXIII, p. 434; 1924–1925, t. LXXI, p. 553.

(6*) A. Carrel et A. H. Ebeling. *Journ. of exper. Med.*, 1923, t. XXXVIII, p. 499.

varier beaucoup la composition des milieux nutritifs pour les fibro-
blastes et les cellules épithéliales (3*).   Ce sont les produits de l'hydro-
lyse incomplète de la fibrine qui donnent les meilleurs résultats (3*).
Nous avons obtenu, grâce à eux, des colonies de fibroblastes dont le
diamètre dépassait 30 mm.   Ces mêmes substances, à une concentra-
tion beaucoup plus faible, peuvent servir à l'alimentation des colonies
de macrophages.

Ces progrès dans la connaissance des milieux nutritifs ont étendu
beaucoup les possibilités de la méthode.   Grâce à eux, il est devenu
possible de maintenir des tissus à l'état de culture vriae dans des
milieux qui ne contiennent aucune substance provenant des animaux
dont les tissus sont originaires.

LE RÔLE DES PRODUITS DE L'HYDROLYSE INCOMPLÈTE DE LA FIBRINE DANS LA PROLIFÉRATION CELLULAIRE.

PAR ALEXIS CARREL ET LILLIAN E. BAKER.

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Dans leur multiplication *in vitro,* les fibroblastes utilisent les premiers produits de la digestion peptique de la fibrine commerciale, ainsi que nous l'avons montré dans une note précédente (1). Nous avons remarqué aussi que parmi toutes les protéines qui ont été hydrolysées, la fibrine détermine la croissance la plus rapide des colonies cellulaires. On dirait que la fibrine partiellement digérée est un aliment de choix pour les cellules.

Comme la fibrine commerciale desséchée est relativement impure et contient de nombreux globules sanguins, nous nous sommes demandé si l'effet particulier qu'elle exerce sur les fibroblastes ne lui appartient pas en propre et provient de quelque impureté. Il était donc indispensable de se procurer cette protéine à l'état pur. C'est pourquoi nous préparâmes de la fibrine à l'aide de sang de Lapin recueilli dans des tubes paraffinés et immédiatement centrifugé et aussi de sang de Bœuf citraté soumis à des centrifugations répétées, afin d'enlever tous les globules. On obtint ainsi du plasma très pur. Et même, dans une des expériences, ce plasma fut passé dans un filtre Berkefeld. On précipita la fibrine du plasma en le saturant à moitié avec du chlorure de sodium. Le précipité fut lavé plusieurs fois dans une solution à demi-saturation de chlorure de sodium, puis dans un grand excès d'eau distillée, afin de la débarrasser aussi complètement que possible des matières étrangères. Les précipités furent digérés par la pepsine jusqu'à ce que le rapport de l'azote total à l'azote aminé soit de 8 environ. On continua la préparation des substances de la façon qui a été déjà décrite (2). On les compara ensuite à celles provenant de l'hydrolyse de la fibrine commerciale, au

(1) A. Carrel. *C. R. de la Soc. de biol.,* 1926, t. XCIV, p. 1060.
(2) A. Carrel et L. E. Baker. *Journ. of exp. Med.,* 1926, t. XLIV, p. 503.

point de vue de leur effet sur la prolifération cellulaire.   Les résultats furent semblables.   Il n'y a donc aucun doute que l'action exercée sur les fibroblastes par la fibrine commerciale hydrolysée ne soit due à la fibrine elle-même, et non pas à des substances provenant des globules sanguins qui la contaminent.

La concentration en azote des solutions de fibrine hydrolysée était de 0,6 p. 100, c'est-à-dire beaucoup plus forte que celle du suc embryonnaire, qui entre généralement dans la composition des milieux de culture.   Au cours d'expériences plus récentes, nous trouvâmes qu'une concentration d'azote six à dix fois plus faible, c'est-à-dire variant de 0,06 à 0,1 p. 100, peut déterminer une croissance des colonies égale à celle produite par les extraits de pulpe embryonnaire dont la concentration en azote oscille de 0,035 à 0,05 p. 100.   La concentration d'azote nécessaire à la croissance des fibroblastes n'est donc pas beaucoup plus grande que celle qui est demandée par les macrophages.   Mais les produits de l'hydrolyse de la fibrine sont toxiques pour les macrophages à une concentration qui n'est pas nocive pour les fibroblastes.

Les colonies de fibroblastes cultivés dans ces solutions purent s'accroître plus rapidement que dans le suc d'embryon et atteindre un diamètre supérieur à 30 mm., ce qui n'avait jamais été observé jusqu'à présent.   Cependant, ces produits n'agissent pas sur les tissus de la même façon que les extraits du pulpe embryonnaire.   Ils déterminent souvent la digestion du coagulum autour du tissu.   Le phénomène se manifeste surtout quand on emploie des cultures pures de fibroblastes.   Dans les cultures de fragments de tissu frais, la digestion ne se produit pas.   La liquéfaction du caillot autour du tissu n'est pas due à une réactivation de la pepsine, car elle s'observe aussi quand la protéine est hydrolysée par de la vapeur sous pression.   Si, au lieu de cellules de Poule, on cultive des cellules de Rat, on n'observe pas la digestion du coagulum.   C'est grâce à cet artifice que nous avons pu étudier l'effet de la fibrine digérée sur des cultures pures de fibroblastes.

Les cellules de Poule cultivées dans des milieux contenant des protéoses et des peptones diffèrent de celles qui prolifèrent dans du suc d'embryon.   En effet, les fibroblastes cultivés dans ce dernier milieu et convenablement lavés montrent un cytoplasme tout à fait

transparent qui contient seulement de très petits globules graisseux. Au contraire, les cellules qui se multiplient dans les protéoses et les peptones ont un aspect très caractéristique à cause de la présence de gros globules de graisse.   En même temps, ces cellules possèdent des prolongements clairs, minces et longs, et manifestent une grande activité.   On peut donc supposer que les produits du dédoublement de la fibrine contiennent quelque substance toxique, ou bien n'apportent pas à la cellule tout ce qui lui est nécessaire pour la synthèse du cytoplasma normal.

Nous avons comparé, au point de vue de leur effet sur les fibroblastes, la fibrine, la caséine, les tissus d'embryon, le tissu musculaire, les glandes thyroïde et pituitaire, le thymus, etc.   Les meilleurs résultats ont toujours été obtenus avec de la fibrine.   Il est certain que les premiers produits du dédoublement de cette protéine déterminent de façon particulièrement énergique la prolifération cellulaire. On peut supposer que, dans l'organisme lui-même, la digestion de la fibrine déterminerait une réponse analogue des cellules.   Il devient alors possible de comprendre le rôle de la fibrine qui, au cours des processus les plus variés, se dépose dans les tissus.   Cette fibrine agirait, no seulement comme un échafaudage dont les cellules se servent au cours de la régénération ou de l'inflammation des tissus, mais comme une réserve d'aliments d'une valeur exceptionelle que, dans certaines conditions, des ferments peuvent libérer.

HYDRATION OF GELATIN IN SOLUTION.

BY M. KUNITZ.

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The high viscosity of gelatin solutions indicates that even at temperatures as high as 40°–50°C. gelatin is hydrated,[1] *i.e.*, that the ultimate gelatin units (particles or molecules) in the solution are in some way combined with a certain amount of water. This is true not only in the case of ionized gelatin, where the hydration is regulated by the Donnan equilibrium, but also in the case of isoelectric gelatin, the viscosity of which, even at high temperatures, is quite high as compared with that of crystallized egg albumin of the same concentrations, as shown in Fig. 1. The hydration of gelatin is indicated also by measurements of osmotic pressure of various concentrations of gelatin solutions. In this respect gelatin differs from egg albumin. The curve, Fig. 2, for osmotic pressure *vs.* concentration for isoelectric albumin (sp. cond. about 8×10^{-5} in 15 per cent sol.) at 20°C. is a straight line, while in the case of gelatin the osmotic pressure increases much faster than the concentration, and the curve is convex toward the concentration axis. This difference in the behavior of gelatin and egg albumin with respect to osmotic pressure is explainable by the difference in their degree of hydration. As was shown elsewhere,[2] the osmotic pressure of dilute molal solutions of hydrated substances may be expressed as

$$P = K \frac{C}{100 - \varphi} \tag{1}$$

where $K = \dfrac{RT}{M}$ (M = mol weight of solute).

C = gm. of solute per 100 cc. of solution.

φ = volume of the hydrated solute,

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[1] Bogue, R. H., The chemistry and technology of gelatin and glue, New York and London, 1922, 194.

[2] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 723.

which means that the osmotic pressure would be proportional to the concentration if the concentration were corrected for the volume occupied by the *hydrated* solute.
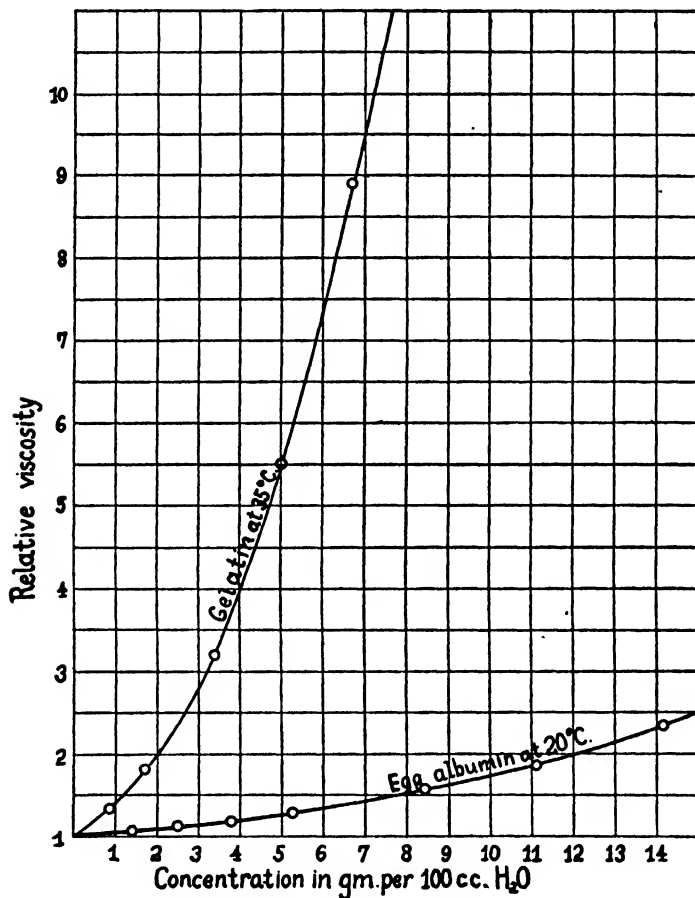
FIG. 1. Viscosity-concentration curves of isoelectric gelatin at 35°C. and of isoelectric egg albumin at 20°C.

In the case of egg albumin the correction is small, and if C is expressed in gm. per 100 cc. of H_2O, there is then practically no correction, and the plotted curve is a straight line. In the case of gelatin, on the other hand, the volume occupied by the hydrated solute is quite large, so that the active concentration of gelatin as expressed in gm.

per 100 cc. of H_2O is much greater than the one taken from dry weight measurements. Hence the osmotic pressure increases much faster than the dry weight concentration. The curves, as said before, should become straight lines if the concentrations of the gelatin solutions were corrected for the volume of the hydrated solute. A means of finding this correction is afforded by viscosity measurements.
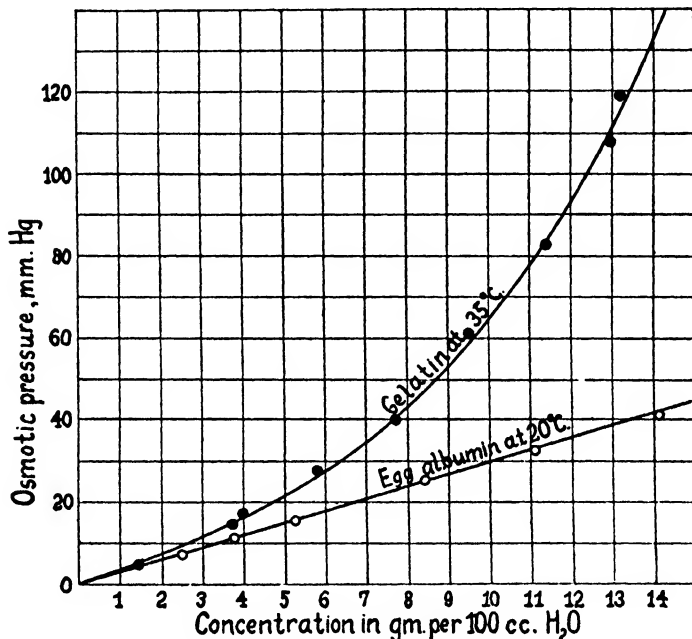
FIG. 2. Osmotic pressure-concentration curves of isoelectric gelatin at 35°C. and of isoelectric egg albumin at 20°C.

A Method for Measuring Hydration of Gelatin.

In a recent publication,[3] the writer showed that the viscosity of a number of colloidal solutions, as well as of various sugar solutions, as measured by means of an Ostwald viscosimeter, may be well represented by the equation

$$\frac{\eta}{\eta_0} = \frac{1 + 0.5\,\varphi}{(1 - \varphi)^4} \tag{2}$$

[3] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 715.

where η is the absolute viscosity of the solution, η_0 is the absolute viscosity of the solvent, and φ is the volume occupied by the solute expressed as a fraction of the total volume of the solution. In the case of sugars or other substances that are hydrated to a very small extent, the value of φ when expressed as cc. per 100 cc. of solution actually equals the volume of the dissolved dry substance, while in the case of highly hydrated or solvated substances, such as casein in
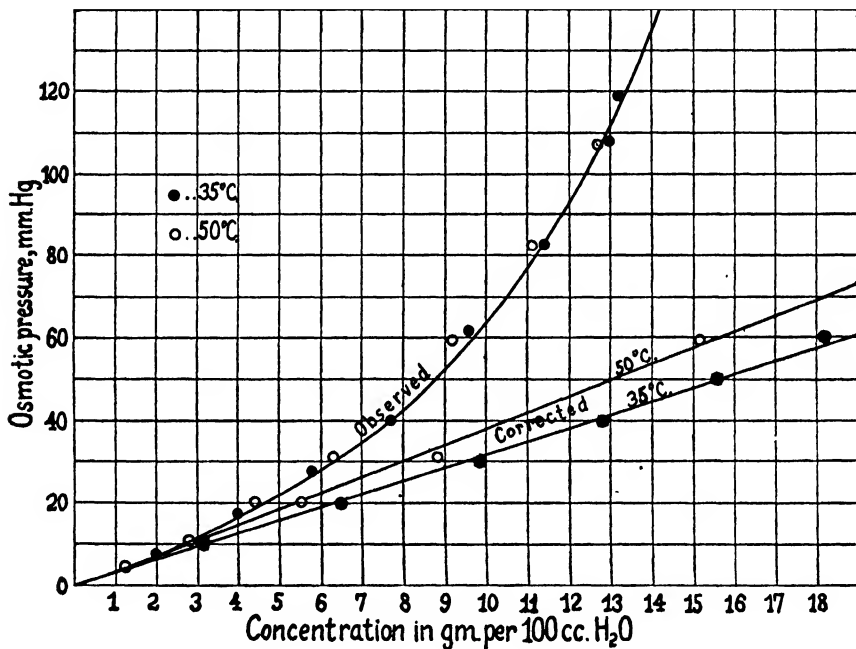
Fig. 3. Effect on the osmotic pressure-concentration curves of gelatin of correcting the concentration of the gelatin for the water of hydration as calculated from viscosity measurements.

water or rubber in benzene, φ represents the volume of the dry substance plus the volume of the solvent associated with it. This was checked for rubber by substituting the values for φ as obtained from viscosity measurements into the equation for osmotic pressure, (equation (1)). The values of K for various concentrations of rubber were constant. The data of Caspari[4] were used in both cases.

[4] Caspari, W. A., *J. Chem. Soc.*, 1914, cv, 2139.

This method of testing the validity of the viscosity formula is applied in this paper to isoelectric gelatin. A series of curves for the osmotic pressure of various concentrations of isoelectric gelatin at various temperatures were plotted. All the curves showed the characteristic convexity toward the concentration axis. The concentrations were expressed as gm. of dry gelatin per 100 cc. H_2O, as determined by drying for 24 hours at 100°C., *definite weights* of gelatin solution taken from the osmometers after equilibrium was reached. At the same time viscosity measurements were carried out by means of an Ostwald viscosimeter on samples of the same gelatin solutions at the same temperatures at which the osmotic pressure measurements were done. The viscosities of freshly prepared gelatin solutions were also measured and gave practically identical results. The various values of φ were then read off from the theoretical viscosity curve shown elsewhere,[5] and the concentrations in the osmotic pressure curves were then corrected. Fig. 3 shows that the corrected points for the osmotic pressure values lie on straight lines in the range of the dry weight concentration from 1 to 10 gm. per 100 cc. of H_2O for the various temperatures used, thus proving that the values of φ as obtained from the viscosity represent the true volumes of the hydrated gelatin particles, and hence affording a method of determining the degree of hydration of gelatin.

Molecular Weight of Gelatin.

One of the difficulties usually experienced in calculating the mol weight of gelatin from osmotic pressure through the application of van't Hoff's formula

$$P = \frac{RT}{M} C \ (C = \text{gm./cc.}),$$

is the fact that P/C, as usually plotted, is not constant. But if, on the other hand, the concentration is corrected for the "water of hydration" by means of viscosity, then RT/M becomes a constant value, expressed as the slope of the corrected curve, or it can be calculated more exactly from the relation

$$\frac{RT}{M} = \frac{P \times (100 - \varphi)}{C} \tag{3}$$

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[5] Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 717.

At 35°C. the value of RT/M is 313, as shown in Table I. Hence

$$M = \frac{RT}{313}$$

R being the gas constant equals

$$\frac{P_0\,V_0}{T_0} = \frac{22.4 \times 760 \times 1000}{273} = 62,400 \text{ cc.mm.Hg/degree}$$

or

$$M = \frac{62,400 \times 308}{313} = 61,500.$$

TABLE I.

C	$\frac{\eta}{\eta_0}$	φ	Corrected concentration $= \frac{C}{100-\varphi}$	P	$\frac{RT}{M}$ uncorrected $= \frac{100\,P}{C}$	$\frac{RT}{M}$ corrected $= \frac{(100-\varphi)\,P}{C}$
gm./100 cc. of solution				mm.Hg		
1	1.43	7.75	1.08	3.5	350	324
2	2.06	15.05	2.35	7.5	375	319
3	2.96	21.80	3.84	12.0	400	312
4	4.24	27.90	5.55	17.0	425	306
5	6.00	33.40	7.50	23.0	460	307
6	8.20	38.10	9.70	29.5	492	304
7	10.85	42.18	12.1	37.5	537	310
8	13.9	45.52	14.7	47.0	588	320
Average..						313

This value represents the weight of dry gelatin which, if dissolved in 1000 cc. of H_2O, will produce an osmotic pressure of $22.4 \times \dfrac{308}{273}$ atmospheres at 35°C.

The Mechanism of Hydration of Gelatin.

The experiments of Loeb[6] on viscosity of gelatin at various pH led him to the conclusion that gelatin solutions contain a number of

[6] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924, 270.

submicroscopic particles of solid jelly which are able to take up water and swell when there is a greater osmotic pressure inside the particles than outside. On the addition of acid or alkali to a solution of iso-electric gelatin a Donnan equilibrium is established between the diffusible ions inside and outside of the particles with the result that, owing to the larger concentration of gelatin inside, there are more ions inside than outside, and the particles then increase in volume on account of the difference in osmotic pressure. According to this view, then, these submicroscopic particles of solid jelly behave with respect to the Donnan equilibrium in agreement with the Procter-Wilson[7] theory of swelling of blocks of gelatin. According to this theory a block of gelatin under the influence of a higher ion activity inside than in the surrounding medium takes up water until the difference in the osmotic pressure between the inside and the outside solution is balanced by the stress in the elastic structure of the block, which appears to obey Hooke's law, *i.e.*,

$$e = E V$$

where e is the osmotic pressure due to the difference in the activity of ions, E is the bulk modulus, and V is the increase in volume.

This theory appears to hold also for the swelling of blocks of *iso-electric* gelatin, as was shown by Northrop and the writer.[8] In the case of isoelectric gelatin, where ions are practically absent, an osmotic pressure exists in a block of solid gelatin due to the presence of a water-soluble constituent of gelatin held in a network of insoluble fibers. This was confirmed by actually isolating from gelatin, by fractional precipitation with alcohol, two fractions, one of which is soluble in cold water, does not set to a jelly, and has a low viscosity and a high osmotic pressure, and a second one which is insoluble at ordinary temperatures, sets to a jelly in very low concentration, and swells much less than ordinary gelatin. When a block of ordinary solid isoelectric gelatin is immersed in water, the water enters the gelatin which swells until the osmotic pressure of the soluble fraction of the

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[7] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307. Wilson, J. A., and Wilson, W. H., *J. Am. Chem. Soc.*, 1918, xl, 886.

[8] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 161.

gelatin inside of the block is equal to the elasticity pressure of the block, *i.e.*,

$$P = E \frac{V_s - V_0}{V_0}$$

where P is the osmotic pressure in the block which can be measured directly as described,[9] E is the bulk modulus of elasticity, V_s is the final volume of the block at equilibrium, and V_0 is the volume of the dry gelatin.

It will now be shown from the viscosity measurements of various concentrations of isoelectric gelatin at 35°C. that the apparent hydration of gelatin at this temperature is due to the swelling of the ultramicroscopic particles of solid jelly brought about by the same mechanism as the swelling of large blocks of gelatin,—namely, by the osmotic pressure of the *soluble* gelatin which is included in the insoluble particles of solid jelly, or micellæ as they were named by Naegeli. The particles swell until the difference between the osmotic pressure inside and outside of the particles is balanced by the elastic pressure of the particle.

Let q be amount of water in cc. held by 1 gm. of gelatin, as calculated from viscosity, *i.e.*

$q = \dfrac{\varphi}{C} - .75$, the last value being the volume of 1 gm. of dry gelatin.

Let a n be the number of micellæ per gm. of gelatin.

$\dfrac{\text{Iso}}{q}$ = cc. H_2O per micella, under the assumption that all the water of hydration is associated with the micellæ only.

s = gm. of soluble gelatin per micella.

K = osmotic pressure constant for soluble gelatin, *i.e.*,

$$K = \frac{RT}{M}$$

Then the osmotic pressure inside at equilibrium equals $\dfrac{Ks}{q/n} = \dfrac{Ksn}{q}$.

The osmotic pressure outside is the osmotic pressure P as determined directly, while the elastic pressure is

$$E \frac{q/n}{v_0} = E \frac{q}{v_0 n}$$

[9] Northrop and Kunitz,[8] p. 162.

where E is the bulk modulus of the micellæ at the given temperature, and v_0 is the volume of the micella before swelling. The equilibrium condition is then

$$\frac{Ksn}{q} - P - E\frac{q}{v_0 n} = 0;$$

or if instead of sn the symbol α is used, where α designates the fraction of 1 gm. of gelatin which is found in a soluble state inside of the micellæ, then the equation becomes

$$\frac{K\alpha}{q} - P - E_1 q = 0, \tag{4}$$

where

$$E_1 = \frac{E}{v_0 n}$$

As the concentration of the gelatin approaches zero, P approaches it likewise, and we have then

$$\frac{K\alpha}{q} - E_1 q = 0$$

$$[P \doteq 0]$$

or

$$\frac{K\alpha}{E_1} = q^2$$

$$[P \doteq 0]$$

If a curve is plotted for the values of q as obtained from viscosity measurements against the concentrations of gelatin used and if the curve is continued until it crosses the q axis, the value of q at the interception may then be introduced into the last equation. The actual value of q^2 as read off from the curve is close to 52. Thus we have

$$\frac{K\alpha}{E_1} = 52.$$

When P is not equal to zero the equilibrium equation becomes

$$\frac{52}{q} - \frac{P}{E_1} - q = 0 \tag{5}$$

or

$$\left(\frac{52}{q} - q\right) + P = \frac{1}{E_1} \tag{5a}$$

The following table shows the value of $1/E_1$ for various concentrations of isoelectric gelatin at 35°C.

TABLE II.

C	P	q	$\frac{1}{E_1} = \left(\frac{52}{q} - q\right) + P$
gm./100 cc. of solution	*mm. Hg*		.
1	3.5	7.00	.123
2	7.5	6.78	.120
3	12.0	6.52	.123
4	17.0	6.30	.125
5	23.0	5.93	.123
6	29.5	5.60	.175
7	37.5	5.28	.122
8	47.0	4.94	.120
Average...			.123

Substituting into equation (5) for $1/E_1$ its value of .123, and for P the relation

$$P = \frac{K_1 C}{100 - \varphi} \text{ (equation (3)), where}$$

$$K_1 = 313^* \quad \text{and} \quad \varphi = (q + .75)C$$

we get

$$\frac{52}{q} - \frac{38C}{100 - (q + .75)C} - q = 0 \tag{6}$$

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* Table I.

Fig. 4 shows the plotted theoretical curve for equation (6), obtained by assuming various values for q and solving for C. On the same figure are also shown the experimental values for q at various values of C, which are very close to the theoretical line. This agreement between the theoretical values of the water of hydration of gelatin with the values obtained from viscosity measurements confirms the theory that a solution of isoelectric gelatin even at 35°C. contains a definite number of small blocks of gelatin filled with a definite weight
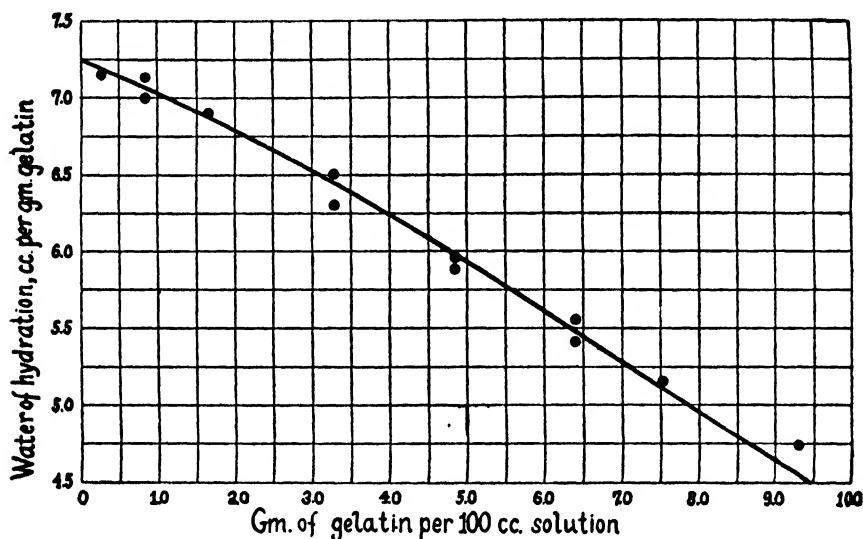
FIG. 4. Relation between the concentration of gelatin and the amount of water of hydration per gm. of dry gelatin. The dots are the values obtained from viscosity measurements. The smooth curve represents the theoretical relation:

$$\frac{52}{q} - \frac{38C}{100 - (q + .75)C} - q = 0.$$

of soluble gelatin the osmotic pressure of which is higher than the osmotic pressure of the gelatin outside of the blocks, $i.e.$ higher than the total osmotic pressure of the gelatin solution as a whole. Owing to this difference in pressure, each little block swells until its osmotic pressure is balanced by the total osmotic pressure of the solution and the elastic resistance of the block to stretch.

As the concentration of the gelatin is increased the total osmotic

pressure of the solution is increased. The number of little blocks is also increased, but the amount of soluble gelatin per block is unchanged, with the result that the difference in osmotic pressure between the inside and the outside of the block continuously decreases with the increase in the total concentration of the gelatin solution. The swelling of the little blocks is thus decreasing gradually as shown on the curve. This is also clear from equation (5a) where $\dfrac{52}{q} - q$ = .123 P. An increase in P must be followed also by an increase in the value of $\dfrac{52}{q} - q$, which is possible only when q is diminishing in its value.

The Quantitative Interpretation of pH Viscosity Curves.

If the equation $\dfrac{K\alpha}{q} - P - E_1 q = 0$ is true for isoelectric gelatin then at any other pH outside of the isoelectric point the equation should be $\dfrac{K\alpha}{q} - P + D.P. - E_1 q = 0$ where P equals

$$\frac{313C}{100 - (q + .75)C},$$

and $D.P.$ is the difference between the osmotic pressure inside and outside of the micellæ due to the difference in the ion activity brought about by the Donnan equilibrium established between the micellæ and the gelatin solution outside of the micellæ. This will be true only if the addition of acid or alkali does not modify the values of α and E_1. That this is the case for HCl, at least, was demonstrated by the fact of the reversibility of the swelling of gelatin in an HCl solution, since on washing away the HCl the swollen gelatin returns to the original volume of isoelectric gelatin.[10] This is also true with respect to osmotic pressure. Hence, if values could be substituted for K, α, and E_1, then it would be possible to calculate the internal Donnan pressure of gelatin at various pH from the viscosity measurements. But, as found above,

$$\frac{K\alpha}{E_1} = 52$$

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[10] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–27, viii, 329.

and

$$\frac{1}{E_1} = .123 \quad \text{or} \quad E_1 = 8.1$$

Hence

$$K\alpha = 420$$

Substituting these values, the equation for the internal Donnan pressure of a solution of gelatin chloride at various pH is then

$$D.P. = P + 8.1\, q - \frac{420}{q} \tag{7}$$

There is also another way of obtaining the approximate values of the internal Donnan pressure of the micellæ; namely, through an analysis of the ion distribution between the micellæ and the outside gelatin solution.

Let H_i = hydrogen ion activity in the micellæ.
$[HCl_i]$ = total HCl concentration in the micellæ.
H_0 = hydrogen ion activity outside.
$[HCl_0]$ = total HCl concentration outside.

If it is to be assumed that the gelatin has only a slight effect on the activity of the Cl ion,[11] and that the concentration of HCl outside of the micellæ is not much different than the average concentration of HCl in the gelatin solution, then according to the Donnan equilibrium relation

$$H_i\, \gamma_{Cl_i}\, [HCl_i] = H_0\, \gamma_{Cl_0}\, [HCl_0]$$

where γ_{Cl_i} and γ_{Cl_0} are the activity coefficients for Cl ion,

or

$$H_i\, [HCl_i] = H_0\, [HCl_0]\, \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} \tag{8}$$

The calculations of Loeb[12] on osmotic pressure of gelatin have shown that the Donnan osmotic pressure of a solution of gelatin in a collodion membrane is equal approximately to the sum of the activities of the

[11] Loeb,[6] p. 50. Hitchcock, D. I., *J. Gen. Physiol.*, 1922–23, v, 387. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924–25, vii, 34.
[12] Loeb,[6] p. 217.

ions in the gelatin solution, as measured electrometrically, multiplied by $RT/1000$. This same pressure is also acting against the Donnan pressure of the micellæ, which in its turn equals the sum of the activities of the ions in the micellæ. Hence the difference in the Donnan pressure is

$$D.P. = \frac{RT}{1000} (H_i \times Cl_i - H_0 - Cl_0) \tag{9}$$

where

$$Cl_i = \gamma_{Cl_i} [HCl_i] \quad \text{and} \quad Cl_0 = \gamma_{Cl_0} [HCl_0].$$

Equation (8) may also be put in the form of

$$H_i \left(\frac{H_i}{\gamma_{H_i}} + [HCl_i] \, C \right) = \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} a \tag{10}$$

where $[HCl_c]$ = equivalent concentration of HCl combined with 1 gm. of gelatin when dissolved in 100 cc. of solvent, C = concentration of gelatin in gm. per 100 cc. H_2O, and $a = H_0[HCl_0]$

or

$$H_i^2 + H_i [HCl_c] \gamma_{H_i} C = \gamma_{H_i} \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} a \tag{10a}$$

If it is to be assumed, as a first approximation, that

$$\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} = 1$$

and that

$$\gamma_{H_i} = \gamma_{H_0}$$

then

$$H_i^2 + H_i [HCl_c] \gamma_{H_0} C = \gamma_{H_0} a \tag{10b}$$

or

$$H_i^2 + H_i [HCl_c] C_1 = a_1$$

where

$$C_1 = \gamma_{H_0} C \quad \text{and} \quad a_1 = \gamma_{H_0} a$$

It will be shown later that of every gm. of gelatin dissolved at 35°C., 0.48 gm. is found in the micellæ. Hence if q is the amount of H_2O of hydration per gm. of gelatin, as obtained from viscosity measurement, the concentration of the gelatin in the micellæ, is $C = \dfrac{.48}{q}$, since the water of hydration is associated, according to theory developed here, with the micellæ only.

At any pH the amount of HCl combined per gm. of gelatin in the micellæ can be obtained from the titration curve of gelatin, since it was shown by experiment that there is practically no difference in the titration curves of the soluble and the insoluble fractions of gelatin. Fig. 5 shows the titration curve for 1 per cent solution of gelatin with HCl. The curve was corrected, from the data in Table IV, for the free HCl, *i.e.* for the HCl required to bring H_2O to the corresponding
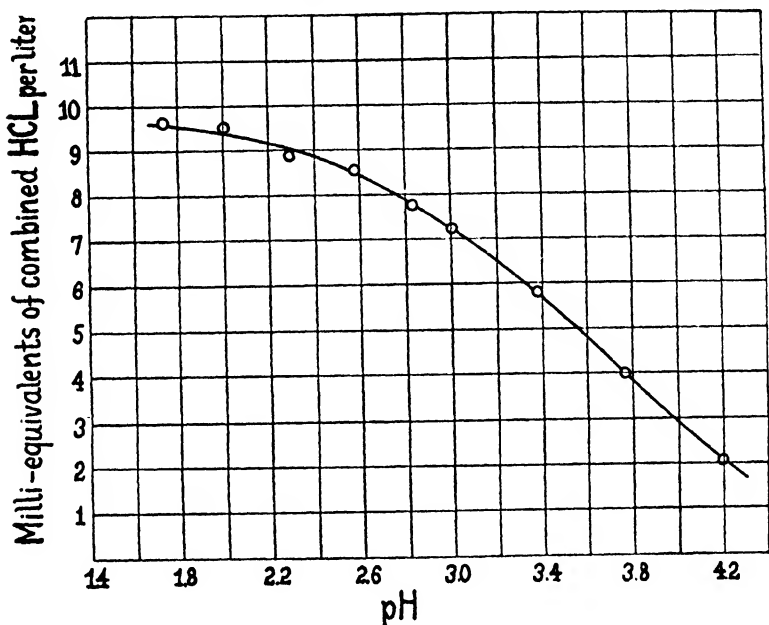
FIG. 5. Corrected titration curve of 1 per cent isoelectric gelatin with HCl.

pH. At any value of H_i the value of HCl_c is thus determinable by reading it off the curve. The correction for the free HCl was based on the relation $[HCl] = \dfrac{H}{\gamma_H} + [HCl_c]C$ where

[HCl] = total concentration of HCl in the gelatin solution.

H = activity of the H ion as obtained from pH measurement.

γ_H = activity coefficient of H at the ionic strength equal to [HCl].[*]

[HCl_c] and C are the same as defined before.

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[*] Gelatin appears to affect neither the ionic strength of a solution nor the activity coefficients of the various ions. See Northrop and Kunitz,[11] p. 29.

Values for γ_H (as well as for γ_{Cl_0}, γ_{H_i}, and γ_{Cl_i}, used in the later calculations) at the various values of [HCl] were calculated, by the method of Lewis and Randall,[13] from the recent data of Scatchard[14] on the mean activity coefficients of KCl and HCl at 25°. The curves for γ_H and γ_{Cl} at various concentrations of HCl are shown on Fig. 6. All pH measurements were done electrometrically at 35°C., and the values for pH were based on 0.100 M HCl as a standard, its pH being
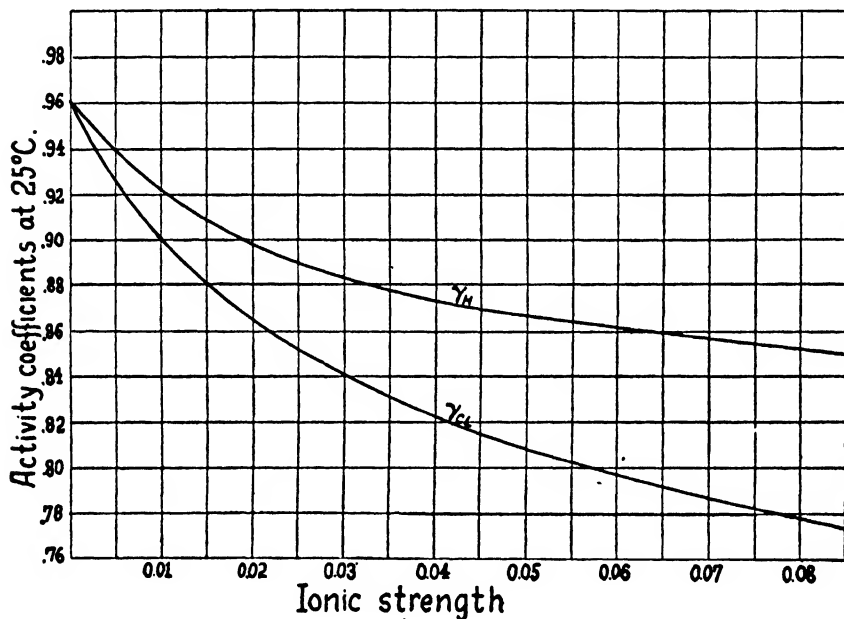
FIG. 6. Activity coefficients for hydrogen and chloride ions at various values of the ionic strength as calculated from the data of Scatchard on the mean activity coefficients of KCl and HCl at 25°C.

taken as 1.085 at 35°. It is thus possible, by means of equation (10b), to calculate approximately the activities of the ions in the micellæ from the known data on the whole solution and from the additional information on the concentration of the gelatin in the micellæ from the viscosity measurements, especially since [HCl.]

[13] Lewis, G. N., and Randall, M., Thermodynamics and the free energy of chemical substances, New York and London, 1923, 381.

[14] Scatchard, G., J. Am. Chem. Soc., 1925, xlvii, 660.

can be easily expressed as a function of H_i by means of the equilibrium equation representing the titration curve. The actual calculations were done graphically by assuming various values of H and solving for a_1 at a given value of C_1. This was repeated for the identical values of H and another value of C_1. A family of curves were then plotted for C_1 of 2.0, 2.5, 3.0, 3.5, and 4.0 of pH as abscissæ and values of a_1 as ordinates. These curves were then used for finding the pH of the
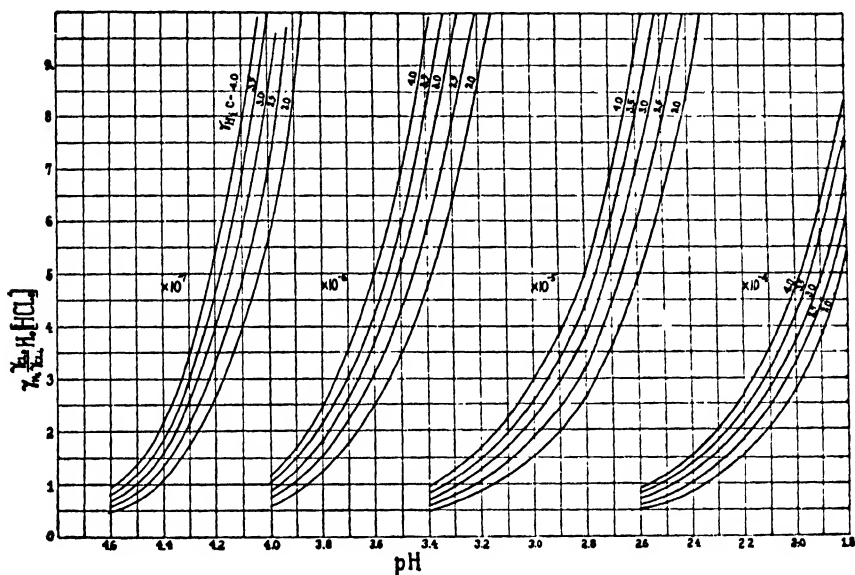
Fig. 7. Auxiliary curves for obtaining the values of pH in the micellæ of 1 per cent gelatin-HCl solution from the relation:

$$H_i^2 + H_i [HCl_i] \gamma_{H_i} C = \gamma_{H_i} \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} H_0 [HCl_0]$$

micellæ for various values of $\gamma_{H_0} a$ and $\gamma_{H_0} C$, thus giving values of H_i. On substituting the found value of H_i in equation (8), and assuming that $\frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} = 1$, the value of $[HCl_i]$ was obtained, namely $[HCl_i] = \frac{a}{H_i}$. Using the obtained approximate values of $[HCl_i]$ as the ionic strength of the solution in the micellæ, values for γ_{H_i} and γ_{Cl_i} were

read off the curves. New values of H_i and $[HCl_i]$ were then obtained in the same manner as before except instead of $a_1 = \gamma_{H_0} a$ and $C_1 = \gamma_{H_0} C$, values of $a_{,,} = \gamma_{Hi} \dfrac{\gamma_{Cl_0}}{\gamma_{Cl_i}} a$ and $C_{,,} = \gamma_{H_i} C$ were employed; also in equation (8) the actual values of $\dfrac{\gamma_{Cl_0}}{\gamma_{Cl_i}}$ were used. The new values of H^i and $[HCl_i]$ happened to be almost identical with those obtained on the first approximation and hence they were taken as the correct ones. The sum of the activities of the ions inside of the micellæ is then $H_i + \gamma_{Cl_i} [HCl_i]$ and outside $H_0 + \gamma_{Cl_0} [HCl_0]$, and the Donnan pressure is

$$19.2 \times 10^3 \times \left(H_i + \gamma_{Cl_i} [HCl_i] - H_0 - \gamma_{C_0} [HCl_0] \right)$$

where 19.2×10^3 is the theoretical osmotic pressure of a molar solution in mm. of Hg at 35°C. The following is an example of the calculations.

A solution of 0.97 per cent gelatin containing 8.13 cc. N/10 HCl per 100 cc. gave a pH reading of 3.01. Its relative viscosity at 35°C. was found to be 2.50 with a value for q of 18.65.

$$[HCl_0] = 8.13 \times 10^{-3}$$

$$\left. \begin{array}{l} \gamma_{Cl_0} = .910 \\ \\ \gamma_{H_0} = .928 \end{array} \right\} \text{At ionic strength of } 8.13 \times 10^{-3}$$

$$H_0 = 9.77 \times 10^{-4} \text{ from pH}$$

$$a = H_0 [HCl_0] = 7.94 \times 10^{-6} \text{ and } \gamma_{H_0} a = 7.36 \times 10^{-6} = a_1$$

$$C = \frac{.48}{18.65} = 2.57 \text{ gm./100 cc. } H_2O \text{ and } \gamma_{H_0} C = 2.39 = C_1$$

pH inside (from curve for $C_1 = 2.5$ and extrapolated for 2.39) $= 3.31$

$$H_i \text{ from } pH_i = 4.90 \times 10^{-4}$$

$$[HCl_i] = \frac{a}{H_i} = \frac{7.94 \times 10^{-6}}{4.79 \times 10^{-4}} = 16.2 \times 10^{-3}$$

$$\gamma_{H_i} = .906 \text{ and } \gamma_{Cl_i} = .878 \text{ for the ionic strength of } 16.2 \times 10^{-3}$$

$$a_{\ell} = \gamma_{H_i} \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} a = .906 \times \frac{.911}{.878} \times 7.94 \times 10^{-6} = 7.46 \times 10^{-6}$$

$$C_{\ell} = \gamma_{H_i} C = .906 \times 2.57 = 2.33 \text{ gm./100 cc. } H_2O$$

Corrected pH$_i$ = 3.30 (from curve for C_{ℓ} = 2.5 and extrapolated for 2.33)

Corrected H$_i$ = 5.01×10^{-4}

$$\text{Corrected [HCl}_i] = \frac{\gamma_{Cl_0}}{\gamma_{Cl_i}} \times \frac{a}{H_i} = \frac{.911}{.878} \times \frac{7.94 \times 10^{-6}}{5.01 \times 10^{-4}} = 16.4 \times 10^{-3}$$

Corrected Cl$_i$ = γ_{Cl_i} [HCl$_i$] = 14.4×10^{-3}

Cl$_0$ = γ_{Cl_0} [HCl$_0$] = 7.4×10^{-3}

Total activity of ions inside = H$_i$ + Cl$_i$ = $5.01 \times 10^{-4} + 14.4 \times 10^{-3} = 14.9 \times 10^{-3}$

Total activity of ions outside = H$_0$ + Cl$_0$ = $9.8 \times 10^{-4} + 7.4 \times 10^{-3} = 8.4 \times 10^{-3}$

Donnan pressure = $19.2 \times 10^3 \times (14.9 - 8.4) \times 10^{-3} = 125$ mm. Hg

This Donnan pressure when calculated by means of the pressure-elasticity equation is

$$D.P. = P + 8.1 \, q - \frac{420}{q} = 3.5 + 151 - 22.5 = 132 \text{ mm. Hg}$$

which is identical, within the limits of error, with the value of 125 as calculated above.

Table III shows the values of the difference in Donnan pressure in mm. of Hg between inside and outside of the micellæ in 0.97 per cent gelatin solution of various pH as calculated by both methods. The same is shown on Fig. 8. These results confirm quantitatively the theory of viscosity of gelatin of various pH, as developed by Loeb, namely, that the viscosity is regulated by osmotic forces due to the Donnan equilibrium. A difference in the activities of the ions inside and outside of the micellæ is established because the concentration of the gelatin in the micellæ is greater than the outside concentration of the gelatin in the solution.

Equivalent concentration of HCl	0	2.03×10^{-3}	4.06×10^{-3}	6.10×10^{-3}
pH_0	4.8	4.20	3.76	3.38
Relative viscosity	1.40	1.62	1.99	2.32
q	6.65	9.85	14.05	17.25
Concentration of gelatin in the micellæ $= \dfrac{.48}{q}$	7.22	4.87	3.41	2.78
H_0 from pH		6.3×10^{-5}	1.74×10^{-4}	4.17×10^{-4}
$Cl_0 = \gamma_{Cl_0} [HCl_0]$		1.9×10^{-3}	3.8×10^{-3}	5.6×10^{-3}
H_i		2.75×10^{-5}	8.5×10^{-5}	2.09×10^{-4}
$Cl_i = \gamma_{Cl_i} [HCl_i]$		4.4×10^{-3}	7.7×10^{-3}	11.2×10^{-4}
Donnan pressure mm.Hg $19.2 \times 10^3 \times (H_i + Cl_i - H_0 - Cl_0)$		42.0	73.0	104
Donnan pressure from relation $K\alpha/q - P + D.P. - E_1 q = 0$ $P = 3.5, K\alpha = 420, E_1 = 8.1$ mm.Hg		41.0	88.0	120

III.

0.97 Per Cent Gelatin-HCl Solution at 35°C.

8.13×10^{-3}	9.14×10^{-3}	11.2×10^{-3}	14.2×10^{-3}	20.3×10^{-3}	30.5×10^{-3}
3.01	2.83	2.58	2.29	2.01	1.73
2.50	2.54	2.52	2.40	2.19	1.99
18.65	18.95	18.85	18.75	16.05	14.10
2.57	2.53	2.54	2.56	2.99	3.40
9.77×10^{-4}	1.48×10^{-3}	2.63×10^{-3}	5.13×10^{-3}	9.8×10^{-3}	18.6×10^{-3}
7.4×10^{-3}	8.3×10^{-3}	10.0×10^{-3}	12.6×10^{-3}	17.6×10^{-3}	25.6×10^{-3}
5.01×10^{-4}	7.59×10^{-4}	1.48×10^{-3}	2.95×10^{-3}	5.98×10^{-3}	12.9×10^{-3}
14.4×10^{-3}	16.1×10^{-3}	17.8×10^{-3}	21.9×10^{-3}	28.8×10^{-3}	36.9×10^{-3}
125	134	128	138	138	107
132	136 ·	135	133	108	88

Effect of Concentration of Gelatin on the pH—Viscosity Curves.

With the increase in the total concentration of the gelatin solution the difference in the gelatin concentration between the micellæ and the outside is gradually diminished.　Hence the increase in viscosity at pH 3.0 over that of isoelectric gelatin, which as shown above is
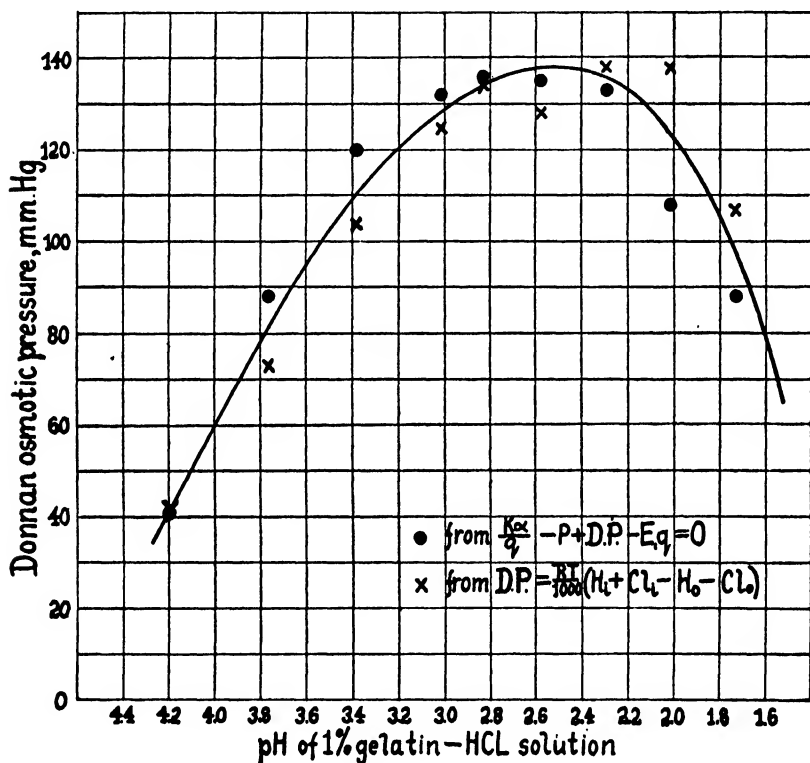
FIG. 8. The difference in the Donnan osmotic pressure between the micellæ and the outside solution at various values of pH in 1 per cent gelatin-HCl solution at 35°C. as calculated from viscosity measurements.

brought about by the difference in the gelatin concentration of the micellæ, should become less conspicuous with increase in the total concentration of the gelatin solution.　That this is exactly what happens is shown in Table IV.

The effect of the concentration of the gelatin solution on the

viscosity-pH curves is shown still more strikingly if instead of the relative viscosity values the values of q, *i.e.*, of the volume of H_2O taken up by a gm. of gelatin as calculated from equation (2), are used in plotting the curves. This is shown on Fig. 9. The enormous effect of the concentration of the gelatin on the viscosity that is caused by the Donnan equilibrium between the micellæ and the outside solution is apparent.

The curves show that at a concentration of 10 gm. of dry gelatin per 100 cc. of H_2O the Donnan effect on the viscosity of the solution disappears entirely. This indicates that at this concentration of gelatin there is no difference between the concentration of the gelatin inside and outside of the micellæ. Let α_1 be the fraction of each gm.

TABLE IV.

Viscosity Measurement of Various Concentrations of Gelatin, pH 4.7 and pH 3.0, at 37°C.

Concentration in gm. per 100 cc. solution	0.5	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
Relative viscosity of gelatin, pH 4.7......	1.16	1.43	1.95	2.75	3.83	5.28	6.70	12.4	21.3
Additional viscosity = relative viscosity − 1..................................	0.16	0.43	0.95	1.75	2.83	4.28	5.70	11.4	20.3
Relative viscosity of gelatin, pH 3.0......	1.84	2.39	3.44	4.54	5.78	7.12	9.06	14.2	22.0
Additional viscosity.....................	0.84	1.39	2.44	3.54	4.78	6.12	8.06	13.2	21.0
Ratio of additional viscosity, pH 3.0/pH 4.7......................	5.24	3.23	2.57	2.02	1.69	1.43	1.42	1.16	1.03

of dry gelatin found in the micellæ, then the concentration of the gelatin inside of the micellæ is α_1/q since q is the volume of H_2O containing α_1 gm. of gelatin. Hence in a solution of gelatin containing 10 gm. of dry gelatin per 100 cc. of H_2O

$$\frac{\alpha_1}{q} = \frac{10}{100}$$

Substituting the value of q, we get

$$\frac{\alpha_1}{4.8} = \frac{10}{100}$$

or

$$\alpha_1 = 0.48$$
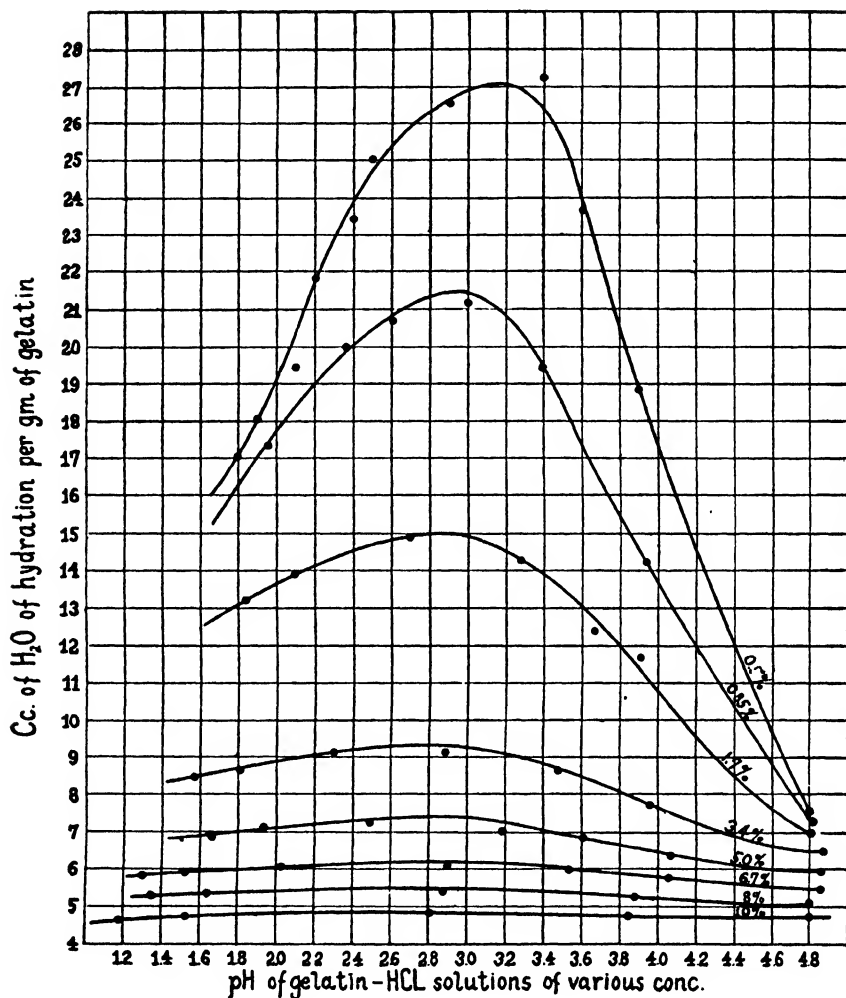
FIG. 9. Effect of concentration of gelatin on the pH-viscosity curves of gelatin-HCl solutions. The viscosity values are expressed in cc. of H_2O of hydration per gm. of gelatin.

This value of α_1 has been used in calculating the concentration of the gelatin in the micellæ of a 1 per cent solution of gelatin at various pH on the assumption that the relation between the fractions of the soluble and insoluble components of gelatin is not affected, within

certain limits, by the total concentration of the gelatin or by the pH of the solution. The agreement between the values for the Donnan pressure in the micellæ as calculated by two independent methods shows that the assumption is correct.

SUMMARY.

1. It was shown that the high viscosity of gelatin solutions as well as the character of the osmotic pressure-concentration curves indicates that gelatin is hydrated even at temperatures as high as 50°C.

2. The degree of hydration of gelatin was determined by means of viscosity measurements through the application of the formula

$$\frac{\eta}{\eta_0} = \frac{1 + 0.5\,\varphi}{(1 - \varphi)^4}.$$

3. When the concentration of gelatin was corrected for the volume of water of hydration as obtained from the viscosity measurements, the relation between the osmotic pressure of various concentrations of gelatin and the corrected concentrations became linear, thus making it possible to determine the apparent molecular weight of gelatin through the application of van't Hoff's law. The molecular weight of gelatin at 35°C. proved to be 61,500.

4. A study was made of the mechanism of hydration of gelatin and it was shown that the experimental data agree with the theory that the hydration of gelatin is a pure osmotic pressure phenomenon brought about by the presence in gelatin of a number of insoluble micellæ containing a definite amount of a soluble ingredient of gelatin. As long as there is a difference in the osmotic pressure between the inside of the micellæ and the outside gelatin solution the micellæ swell until an equilibrium is established at which the osmotic pressure inside of the micellæ is balanced by the total osmotic pressure of the gelatin solution and by the elasticity pressure of the micellæ.

5. On addition of HCl to isoelectric gelatin the total activity of ions inside of the micellæ is greater than in the outside solution due to a greater concentration of protein in the micellæ. This brings about a further swelling of the micellæ until a Donnan equilibrium is established in the ion distribution accompanied by an equilibrium in the osmotic pressure. Through the application of the theory developed here it was possible actually to calculate the osmotic pressure difference

between the inside of the micellæ and the outside solution which was brought about by the difference in the ion distribution.

6. According to the same theory the effect of pH on viscosity of gelatin should diminish with increase in concentration of gelatin, since the difference in the concentration of the protein inside and outside of the micellæ also decreases. This was confirmed experimentally. At concentrations above 8 gm. per 100 gm. of H_2O there is very little difference in the viscosity of gelatin of various pH as compared with that of isoelectric gelatin.

HEART DISEASE FROM THE POINT OF VIEW OF THE PUBLIC HEALTH.*

By ALFRED E. COHN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

The subject of heart disease has become a matter of great public interest since it was pointed out a few years ago that the death rate on this account seemed to be rising. It soon became established that a rise has actually taken place. The experience with tuberculosis and with other infectious diseases suggested the idea that assiduous attention to heart disease like that which had been devoted to these other ailments might yield similar reductions in morbidity and mortality. Associations for the prevention and relief of heart disease were accordingly formed. It was only later, in more recent years, that certain facts began to be subjected to more searching analysis in order to learn, especially from the point of view of prevention and relief, what actually is the situation in respect to this group of diseases. For it required no great amount of reflection to appreciate the fact that, unlike tuberculosis or any other disease of bacterial origin, heart disease, or what went by this name, was composed not of a single entity but of diseases etiologically as distinct as the acute rheumatic affections, syphilis and arterial degeneration. At first it was not clear, and indeed in a general way it is not clear now, either in this or in point of fact in other connections, that arterial decay and degeneration of the heart might not be diseases but the expression of the senescent process. To give expression to the underlying facts we shall in the end, perhaps, be obliged to make a distinction between the decrepitude of old age, a normal and natural growth process, and diseases in old age. That this distinction has importance in the problem I am to discuss, considerations later to be urged may make apparent.

* Delivered as a De Lamar Lecture at the School of Hygiene and Public Health, Johns Hopkins University, Baltimore, November 15, 1926.

When the increase in diseases in connection with the circulation became evident it was natural to compare the curve of death rates as the result of chronic diseases with that of the total death rate curve.

Fig. 1 illustrates the result of this comparison in New York City for the years 1868–1921.[1] At the beginning of this period the ratio of deaths from chronic diseases to the total death rate was about as 1 is
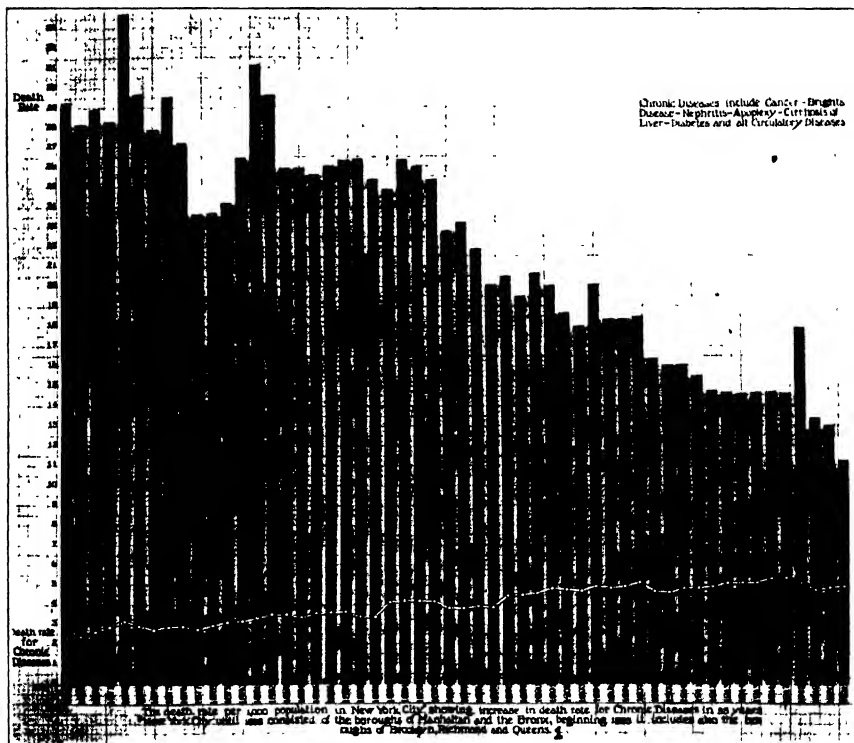
FIG. 1.

to 15. At the end of this period the ratio was about as 1 is to 2. It appears, therefore, that the chronic diseases have taken on a quite new importance. The reason for this is seen in part in studying during the same period of time the changes in death rate which have taken place in certain acute diseases, such as diphtheria, scarlet fever, whooping cough and typhoid fever as exemplified in New York City. (See Fig. 2.)

After 1882 the curves of all of these diseases began to fall and after 1894, when antitoxin against diphtheria was first used, to fall significantly. It is perhaps worth pointing out that the fall occurred even in those diseases against which there was no specific antidote.

Chart showing decrease in death rate per 100,000 population in New York City for Typhoid – Diphtheria – Whooping Cough and Scarlet Fever during the past 53 years
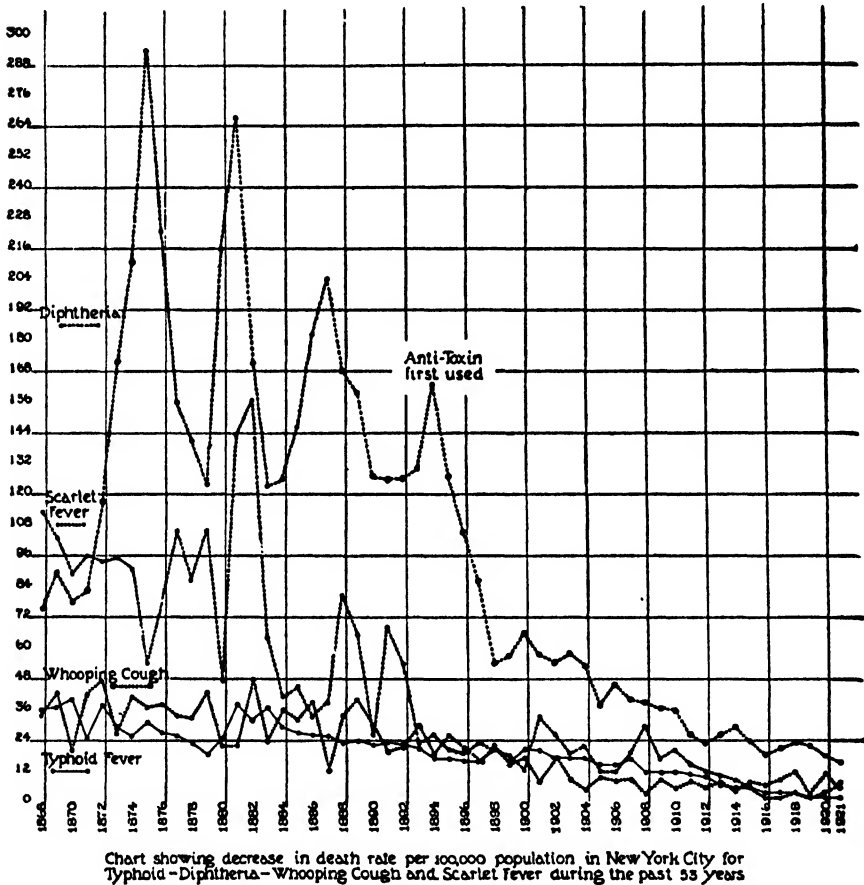
FIG. 2.

That the fall in infectious diseases might influence the death rate movement in heart disease also requires consideration. (See Fig. 3.)*

* I am much indebted to Miss Alice Whittemore for accumulating the data on which this curve is based and for arranging these curves as well as those in Fig. 4.

That the figures before 1900 are unreliable, due to the many phases of faulty technic employed in collecting them, is notorious. For this reason they probably fail to exhibit the course of events and can do no more than represent an approach to the facts. This is unfortunate in view of the important inferences which could otherwise have been drawn. It is, however, important to call attention to the inferences which are implied in considering them.

Before 1896, the curve C of chronic circulatory diseases exclusive of acute circulatory disease rose gradually. For this rise I have as yet no explanation to offer. In the moving average of this curve in the year 1896 there is a sharp inflection, the section afterward showing an abrupt rise. The foot point of the rise is referable probably to the year 1898 in curve C. The high point of the curve A B D of infectious diseases is found seventeen years earlier, in the year 1881. I entertain the belief, as do many others, that the fall in infectious diseases is intimately related with the rise in the cardiac death rate. But I hesitate to state that precisely seventeen years are required after the beginning of the fall of the curve of infectious diseases before a beginning of the rise of the circulatory curve may take place. I should, in any case just now, be at a loss to suggest a proper explanation for the occurrence of exactly this interval. Perhaps the collection of the reports on which these curves are based was open to too many errors

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EXPLANATION OF FIG. 3.

Diseases grouped as follows:

A—(Infectious diseases)
  Typhoid fever
  Measles
  Scarlet fever
  Whooping cough
  Diphtheria
  Influenza
  Mumps
  Dysentery
  Acute poliomyelitis
  Meningococcal meningitis

Malaria
Cholera nostras

B—
  Tuberculosis
  Pneumonia
  Bronchopneumonia

C—(Circulatory diseases exclusive of acute cardiac diseases)
  Apoplexy
  Angina pectoris

Other diseases of the heart (including endocarditis)
Diseases of the arteries
Other diseases of the circulatory system

D—(Acute cardiac diseases)
  Pericarditis
  Acute endocarditis and myocarditis

States included: Connecticut, Indiana, Maine, Massachusetts, Michigan, New Hampshire, New Jersey, New York, Rhode Island, Vermont, and District of Columbia.

MORTALITY RATES PER 100,000 OF VARIOUS DISEASES IN FIRST TEN REGISTRATION
STATES PLUS DISTRICT OF COLUMBIA. 1868–1923.
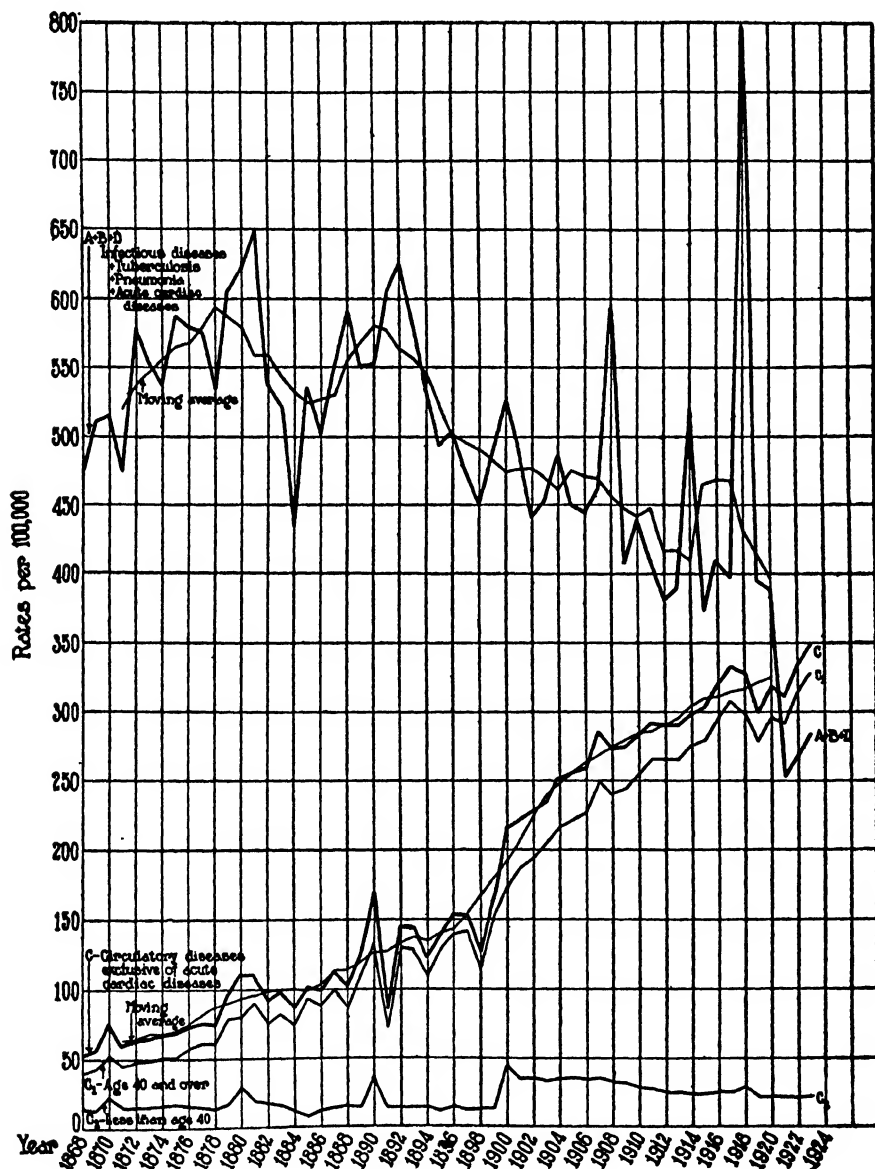
FIG. 3.

of diagnosis or of recording to make valuable the inferences which the curves so clearly suggest. But it seems, taking the general movement of the two curves into consideration, that being saved from infectious diseases preserves the population for exposure to death from more chronic or, from the viewpoint of the aging process, more natural death. It would be rash however to assert that the second process begins seventeen years after the decline of the first.

Infectious diseases may be divided into two groups, in one of which are included tuberculosis and pneumonia and in the other, the other acute infectious diseases as is shown in Fig. 4. It appears, as a result of making this arbitrary division, that if the acute infectious diseases had a share in bringing about the rise in the death rate of circulatory diseases, little influence may be attributed to pneumonia and tuberculosis, for the fall in the curve of these two diseases does not occur until later. It must in consequence be the other infectious diseases, represented in curve *A D*, which may be associated with the change.

These relations tell the story of the changes which took place in heart disease when compared with mortality in general and with certain acute infectious diseases, but it does not suggest its relative importance in connection with other major causes of death such as pulmonary tuberculosis, pneumonia and cancer.

How heart disease increased during the twenty-year period from 1900 to 1920, how the rate from pulmonary tuberculosis fell, how the rate from pneumonia has not reached that of heart disease since 1910 and how cancer is mounting but is even now relatively speaking a small problem numerically compared to heart disease is illustrated in Fig. 5. One detail is indicated here to which I wish especially to direct your attention. You will find it in the heart disease group in the 1920 column. The point I refer to is that, of the total death rate, the proportion which occurs under the age of forty is relatively small, indeed only one-sixth.

That the high rates from heart disease which are encountered in the United States and in large cities like New York are not characteristic of this country, a comparison with other nations and with other cities makes clear. (See Fig. 6.)* In studying data such as those I am

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* I am indebted to Miss Claire Lingg of the Heart Committee of the New York Tuberculosis and Health Association for the data in this figure.

MORTALITY RATES PER 100,000 OF VARIOUS DISEASES IN FIRST TEN REGISTRATION
STATES PLUS DISTRICT OF COLUMBIA. 1868-1923.

FIG. 4.

Diseases grouped as follows:

A—(Infectious diseases)
    Typhoid fever
    Measles
    Scarlet fever
    Whooping cough
    Diphtheria
    Influenza
    Mumps
    Dysentery
    Acute poliomyelitis
    Meningococcal menin-
      gitis

Malaria
Cholera nostras

B—
    Tuberculosis
    Pneumonia
    Bronchopneumonia
C—(Circulatory diseases
    exclusive of acute
    cardiac diseases)
    Apoplexy
    Angina pectoris

Other diseases of the
    heart (including en-
    docarditis)
Diseases of the arter-
    ies
Other diseases of the
    circulatory system
D—(Acute cardiac dis-
    eases)
Pericarditis
Acute endocarditis and
    myocarditis

States included: Connecticut, Indiana, Maine, Massachusetts, Michigan, New
Hampshire, New Jersey, New York, Rhode Island, Vermont, and District of
Columbia.

about to present, I must repeat the warning that they do no more than suggest what sort of information should be available, that they are indeed removed from the possibility of being accurately representative. This state of affairs is, of course, deplorable; one of my purposes, however, consists in indicating how inadequate the data are and how important it is to suggest the directions in which improvement is desirable.

Death rate in ten original registration states
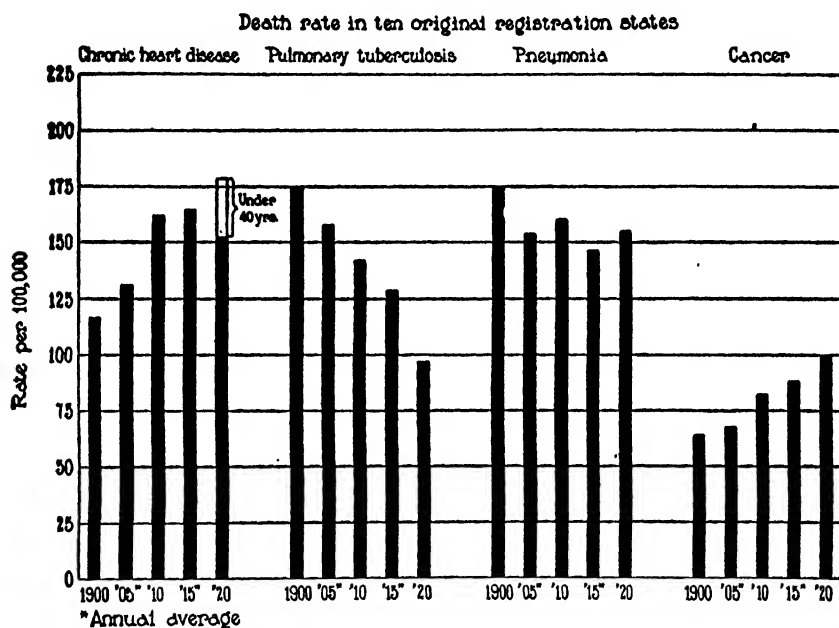
FIG. 5.

For the year 1923 the rate in Germany, 175 per 100,000, is the same as that in the United States. England and Wales and New Zealand are not far removed. If there were time it would be interesting to discuss the low rate in Japan. A consideration of the rates in the cities shows that although the New York rate is not far removed from the median, and that Berlin, New Orleans, and London all show higher rates, its rate as well as that of these cities is greater than that of the United States Registration Area. The same observation applies to Berlin and London in respect to Germany and to England and Wales.

The question has often been raised whether latitude and longitude
and climate influence the incidence and, in consequence, the death
rate from cardiac diseases.   In the United States, if a report is made
according to the geographical division of the states, it appears that

Death rate per 100,000 population (actual or estimated)
for heart diseases – Code Nos-87-90
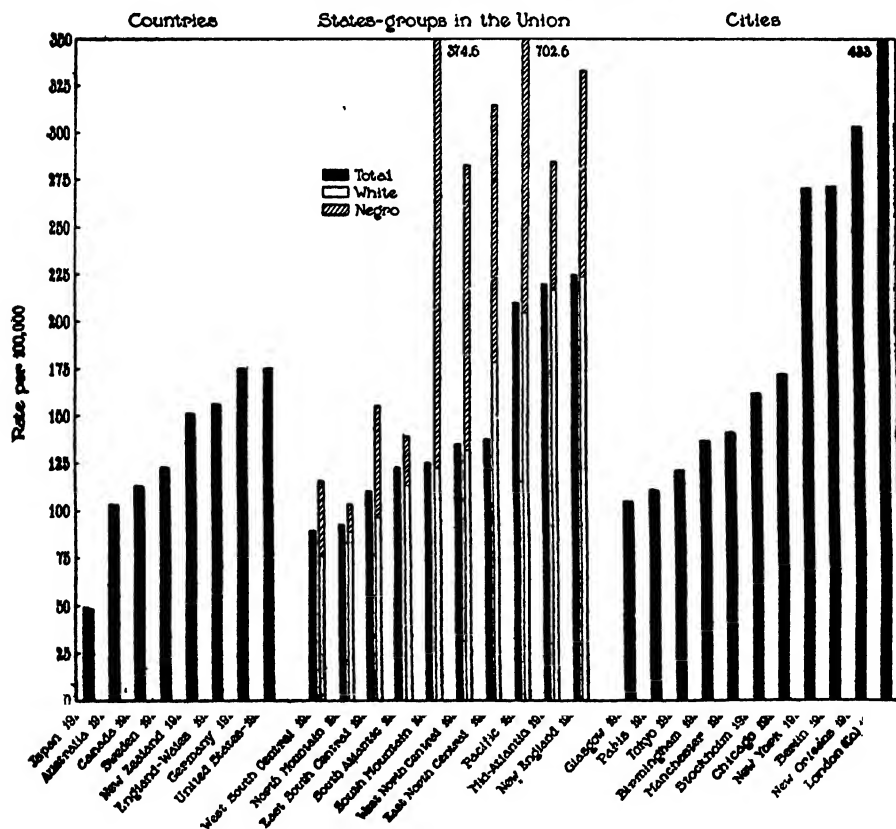
FIG. 6.

the Southern and Mountain States at the left of the middle group of
columns, present distinctly lower rates than the Pacific, New England
or the Middle Atlantic States as is shown in this illustration.   Similar
relations are obtained by comparing death rates from *rheumatic fever*
rather than from heart disease in various countries.   (See Table I.²)

TABLE I.

Rheumatic Fever in Various Countries and Cities.*

Death Rate per 1000 Deaths.

Station	Year	Range of years	Rate per 1000 deaths	Average rate	Range Rate Max. Min.	Range Year Max. Min.	
1. Str. Settlements	1904		0.27				Whole population. All nationalities
2. Honduras	1903		1.01				Whole population Europeans only
3. Jamaica	1903		1.64				
4. N. S. Wales	1903		4.48				
5. England and Wales	1903		4.62				Whole population. All forms of rheumatism
6. Ceylon	1903		6.78				
1. Denmark		1894–1903		1.10	1.42–0.71	1897–1902	
2. Italy		1894–1902		1.21	1.38–1.06	1899–1894	
3. Prussia		1894–1902		2.37	2.57–2.22	1902–1898	
4. Norway		1894–1903		2.86	3.11–1.98	1902–1898	
1. Paris		1901–1903		1.91	2.14–1.75	1903–1902	
2. Christiania		1894–1904		2.35	3.55–1.29	1903–1904	
3. Berlin		1894–1904		2.38	3.53–1.42	1900–1894	
4. Copenhagen		1894–1904		3.23	5.02–0.92	1897–1902	
5. New York City and Brooklyn		1899–1904		3.48			

*From W. S. Church. Rheumatic Fever. Allbutt & Rolleston's "System of Medicine," ii, 594–645.

I must point out, however, that to quote death rates from rheumatic disease is very different from quoting death rates for heart disease for although, especially in the younger age groups, rheumatic fever is a common cause of heart disease, there is no fixed ratio between its occurrence and the number of individuals who develop heart disease.

It appears, for example, that the rate from rheumatic fever is especially low in the Straits Settlements, Honduras, and Jamaica but high in New South Wales, England and Wales, and Ceylon.   In the Straits Settlements, Honduras, and Ceylon the rates are calculated on the basis of the whole population, whereas in Jamaica on that of Europeans only.   In the British colonies the low rates in the tropics may of course in part be due to the British custom of sending children to school in England at just those years at which the incidence of rheumatic fever is highest.   In connection with locality there seems good reason for believing that rheumatic fever is more prevalent in Boston (295 cases or 1.85 per cent of admissions to the Peter Bent Brigham Hospital) than in Oklahoma City (14 cases or 0.1 per cent of admissions to the State University and to St. Anthony's Hospitals[3]).   In the same cities and hospitals mitral stenosis, a disease which is postmortem easily distinguishable and is regarded as a peculiarly characteristic rheumatic disease, occurred in 4.69 per cent of 1362 autopsies and in none of 383 autopsies respectively.   A similar observation has been published on the difference between the University of Virginia Hospital and the Massachusetts General; in the former chorea and rheumatic fever represented 0.7 per cent of admissions, the latter 1.3 per cent.[4]   In comparison, however, with all these figures it is striking that in Northern countries, such as Denmark, Italy, Prussia, and Norway the rates are almost as low as in the tropics.   And in point of fact as great a range in rates is found in the northern cities in Europe and in the United States.   The influence of latitude in any case is not clear and requires further and more detailed studies.   In this connection the influence of season on deaths has also been explored.   In London, for example, the seasonal influence on heart disease has been studied over a period of many years.   (See Fig. 7.) It appears in the second curve quite clearly that in the winter months the number of deaths uniformly rises synchronously with those of the respiratory infections, but curiously enough not with those of influenza.

In New York a similar record has been kept since 1914. (See Fig. 8.) The curves from the point of view now being considered resemble those of London but show a closer relation between deaths from heart disease and from influenza. There are added here curves for tuberculosis and for cancer.

So far, I have dwelt on the place of heart and circulatory diseases in their relation to mortality. The matter is difficult enough to ana-
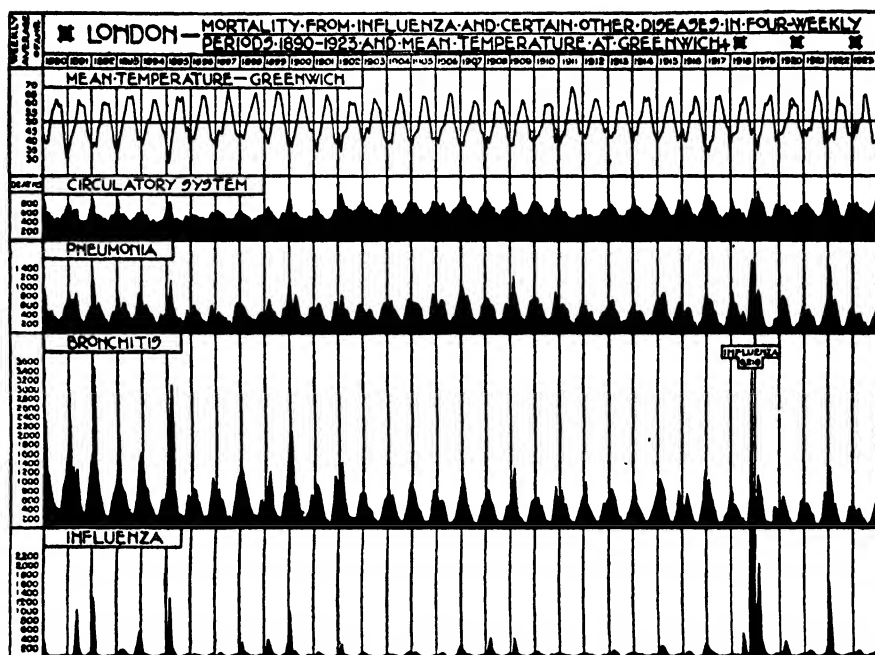
FIG. 7.

* See reference No. 5.

lyze from this angle. But it should be analyzed also from the point of view of morbidity. Here the difficulties, however, increase. I need scarcely point out that I refer to such matters as inaccuracy in diagnosis and incomplete and erroneous reporting, not to mention differences in definition and in classification which are used in various localities. On the whole, though, these diseases are so characteristic that they do not easily escape detection, and the custom which has

Mortality in New York City from chronic heart disease, all respiratory diseases, cancer and pulmonary tuberculosis

Fig. 8.

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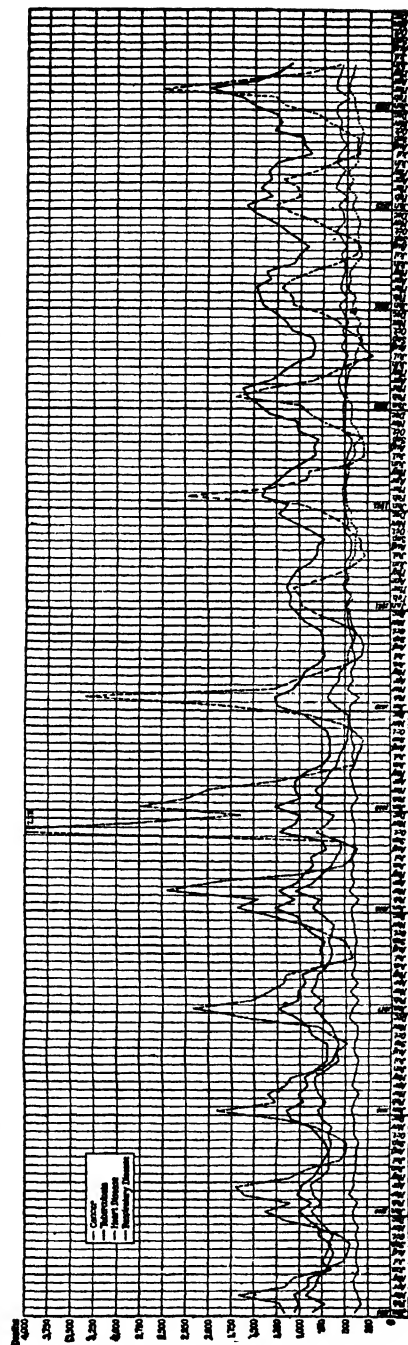
been introduced of reporting in accordance with the International List of Causes of Death has reduced the trouble and has made it possible to place some reliance upon the published figures. From a study of certain morbidity statistics the great importance of the heart diseases also emerges. Data may be drawn from sources so varied as the draft in the United States and in England during the Great War; from statistics such as those of life insurance companies and of the British Insurance Act; from occupations, such as that of newsboys; from the incidence of heart disease in schools both here and abroad; as well as from hospital statistics. (See Table II.) Clearly these are in each case samples only of the population, which need not necessarily be characteristic, as Greenwood and Thompson[6] point out in the case of hospitals.

For the moment, however, data such as these represent the most reliable sources of information. We begin to emerge from a time, not long back, when hospital statistics were the staple in this field. In the United States draft during 1918, of 2,510,791 men, 85,143 or 26.26 per 1,000 were found to have valvular disease of the heart. Among Scottish troops during 1916–17, "whilst still fairly representative," the rate was similar, being 24 per 1,000. Among 1,000 recruits examined for the United States army at New York City in 1926[8] the rate was 15 per 1,000. Data are quoted by Miller to show that there is a difference in incidence between country and city. In the Channel Islands among recruits in 1901–1910, the rate was 1.0 per 1,000 as against 32.4 per 1,000 in the East Riding of Yorkshire. And in Wales during the Great War, among youths eighteen years old, the rate in Carmarthen was 2.0 and in Cardiff 22.0 per 1,000. In the United States on the other hand such striking differences are not discernible. There is no difference between agriculturists and manufacturers in the North, but there is a higher incidence in the North as a whole than in the South; and the rate among negroes in the South is higher than among whites.

Dublin gives 20 per 1,000 as the number of persons rejected each year by the life insurance companies on account of heart disease, whereas Hoffman puts the figure as 24.4. In a report recently made by the Ministry of Health for England and Wales, when 10,476,000 people between sixteen and forty-four years of age appeared on the

TABLE II.

Morbidity.

Chronic Valvular Disease in Various Places and Various Groups.

	Location	Year	Population involved	Heart disease		
				No.	Period	
Troops	U. S. Draft Agriculturists	1918	2,510,751	85,143	26.26	Love and Davenport, 1919[7]
	U. S. Draft. North—White	1917–1918	10,005		34.15	Love and Davenport, 1919, Table 142, p. 281[7]
	U. S. Draft. South—White		10,571		24.60	Love and Davenport "
	U. S. Draft. South—Negro		5,239		29.07	"
	Manufacturing					
	U. S. Draft. East		7,934		35.56	" " "
	U. S. Army	1926	1,000	15	15.00	Munly, Personal communication[8]
	Scotland	1916–1917	10,000	243	24.00	Report No. 23, Ministry of Health, 1924[9]
	Channel Islands	1901–1910			1.0	Kennedy[10]
	Yorkshire. East Riding	1901–1910			32.4	
	Wales. Carmarthen				2.0*	R. Miller, From report on physical examination of men of military age, i, 154, London, 1920[11]
	Cardiff				22.0*	
Insurance	Met. Life Insurance Co.	1915–1918			20.0†	L. I. Dublin, 1925[12]
	Prudential Insurance Co.				24.4†	F. L. Hoffman, 1920[13]
	England and Wales	1922	10,476,000‡	15,912‡	1.51	Report No. 23, Ministry of Health, 1924[9]
Industry	Garment workers				20.0	Schereschewsky, 1920[14]
	Various, Cincinnati				20.0	Robinson and Wilson, 1920[15]
	Food handlers				20.0	Harris and Dublin[16]
	Newsboys—New York City	1925	1,078	15	15.0	N. Y. Tuberculosis and Health Association, 1926[17]

* At age 18; † Of persons who apply for insurance; ‡ Of persons actually insured. This equals the estimated number at ages 16–45.

panel, it was estimated on the basis of actual attack rates that 15,912 might develop rheumatic heart disease if the rate were 1.51 per 1,000 as it appeared to be.   There is apparently a great difference between the rate in the companies and that on the panels.   It must be remembered that the former is, in a sense, an unselected, the latter a selected sample of the community.

In industrial workers in general and in the investigation of garment workers in New York it was found by Schereschewsky[14] that the rate was 20 per 1,000.   Robinson and Wilson[15] arrived at the same figure in various industries in Cincinnati, and in a study of food handlers in New York by the Department of Health, Harris and Dublin[16] found exactly the same figure.   In newsboys in New York studied by the New York Heart Committee the rate was 15 per 1,000.[17]

Lower rates are found among school children.   (See Table III.) But this is not surprising for the ages involved are lower.   Although the age of maximum incidence of rheumatic fever is five to seven years, all children destined to suffer from chronic heart disease have not yet become affected, nor indeed does rheumatic fever take its entire toll at so early an age.   Even before fifteen years of age, it makes a difference whether the rates refer to entrants or to leavers; that for leavers in Glasgow, Bath, and London being twice as great as for entrants.   In England there appears to be a difference also in school children between country and city, for the rate in the rural population is 2.6 per 1,000 and in the industrial 8.0, in a sample of 100,000 school children about thirteen to fourteen years old.

It appears then on the whole that among recruits, applicants for life insurance, and in industry, the incidence of chronic heart disease ranges from 15 to 35.56 per 1,000, the average being 20.8.   There seems moreover to be a difference so far as locality and industry are concerned, for Kennedy gives the rate as low as 1 per 1,000 in the Channel Islands, against 32.4 in the East Riding Yorkshire, and in a country district in Wales as 2 per 1,000 against 22 in Cardiff.

Among school children the range of affection appears to be uncommonly wide, from 3.2 in Lanarkshire to 40 in Rochester, N. Y.   Although the range is wide, in the sixteen localities nine fall between 7 and 16; two below, and five above these figures.   It seems to be so great as to render unlikely the possibility of extracting from its

TABLE III.

Morbidity.

Chronic Valvular Diseases in Various Places Among School Children.

Location	Year	Population involved	No.	Rate per 1000	Entrants 5-6 yr. M.	F.	Intermediate 8 yr. M.	F.	Leavers 12-14 yr. M.	F.	Reference
Lanark		32,000		3.2							G. A. Allan, 1926[18]
Philadelphia		23,671		6.3							Bd. Pub., 1924[19]
Transvaal		30,000		7.0							L. Leipoldt, 1920[20]
Glasgow*		240,000		7.0							G. A. Allan, 1926[18] (includes Aberdeenshire and Edinburgh)
Hempstead				8.2							L. Leipoldt, 1920[20]
Staffordshire		70,138		9.6							L. Leipoldt, 1920[20] (quoted from Priestly)
Aberdeenshire				10.0							G. A. Allan, 1926[18]
England and Wales		266,000		10.0							Report No. 23, Ministry of Health[9]
Glasgow*				10.0							G. A. Allan, 1926[18]
Edinburgh				13.0							G. A. Allan, 1926[18]
New York City		250,000		16.0							S. J. Baker, 1921[21]
Germany				23.0							L. Leipoldt, 1920[20] (quoted from unspecified German statistics)
Bath*	1923	197,327	5,208	23.6							R. Miller, 1926[22]
London	1919	195,162	6,622	26.0							Annual Report, London C. C., 1923[5]
London*	1924		2,400	34.0							Report No. 23, Ministry of Health[9]
Rochester				40.0							A. D. Kaiser, 1926[23]
Glasgow		8,000			5.0				10.0		G. A. Allan, 1926[18]
Bath	1919				10.0				23.6		R. Miller, 1926[22]
London		195,162	6,622		25	22	33	36	38	49	Report No. 23, Ministry of Health[9]
Rural	1924	100,000					Total 34		2.6		R. Miller (quoted from Annual Report of Chief Medical Officer, Board of Education, 1924, London, 1925, 35), B. M. J., 1926[23]
Residential									5.9		
Industrial									8.0		

consideration unified results. It is necessary to be quite certain that the methods of examination were uniform and the criteria for diagnosis fixed and well observed before it is possible to regard the data with confidence. Not to do so would cause the inference to be drawn that heart disease and its contributing etiological factors were prevalent in and affected the several school populations in variety and in numbers too widely different. I refrain therefore from averaging results, apparently so dissimilar.

There appears the fact, then, from a consideration of all the morbidity figures quoted so far that roughly 20 per 1,000 individuals are affected with heart disease in the adult population in the United States. Among school children so narrowly limited a rate cannot yet be given. The adult morbidity figures, however, harmonize even when obtained from such various sources. Is there a correspondence between the morbidity and motality rates? If the figure for the death rate for 1920 in ages below forty years is recalled it was found to be 26 per 100,000; the morbidity rate in the draft was 26 per 1,000; Hoffman's figure for rejections from the Prudential Insurance Company is not far removed from this; that in the industries, namely, 20 per 1,000, is somewhat lower. On the whole the morbidity rate is 100 times greater than that of mortality. In school children the morbidity rate in certain localities corresponds with the adult rate though it is in general much lower. It remains for further investigation to smooth out these disparities.

If we turn now from morbidity statistics of the population at large to consider the number of cases of heart disease which actually arise in medical practice the following figures are discovered. I confine myself to England and to the United States. In the London Children's Hospitals[24] the per cent is 31; Miller[25] gives 38 per cent; Church,[2] 57.5 per cent; St. Lawrence,[26] 61 per cent; Ingerman and Wilson,[27] 69 per cent; Cohn and Swift,[28] 94 per cent. If there are, according to Swift,[29] 175,000 cases of rheumatic fever in the country and if the average of 65 per cent (an average of St. Lawrence's, Ingerman's, and Wilson's rates) of cardiac involvement is accepted for the moment, there should be 113,750 cases of rheumatic heart disease. On the basis of the death rate, one-sixth of cases of heart disease die under forty years of age. Of the age of forty years, there will be discussion presently.

On the assumption that all patients with rheumatic heart disease and only patients with rheumatic disease die under forty, a not unreasonable assumption since the number of cases of syphilis under forty probably balance the number of cases of rheumatic disease above forty, the total cases of heart disease, seeing that cases of rheumatic disease number 113,750, should be six times as many, or 682,500. But the figure usually given for heart disease in the United States is 2,000,000, an estimate which is supported by having found the rate in various circumstances to be 20 per 1,000. There is then a serious error, a difference between 2,000,000 and 682,500, in the assumptions. Or again, if there are 113,750 cases of rheumatic heart disease, they represent only 5.7 per cent of the estimated total of 2,000,000; whereas it is known that one-sixth or 15 per cent of the cardiac population dies before the age of forty. The disagreement between 5.7 per cent of rheumatic cardiac cases and 15 per cent of cardiac deaths before forty is great. If total cardiac disability were 1,000,000 instead of 2,000,000, rheumatic cardiac patients would represent 11.3 per cent instead of 5.7 per cent of the total and would approach the figure for deaths under forty, 15 per cent, more closely. Just now it is impossible to say whether the error is due to too small an estimate in the number of rheumatic cases, to placing the involvement of the heart in rheumatic fever at too low a figure, or perhaps to too high an estimate of the number of persons believed to suffer from heart disease.

The next problem, after having outlined from mortality and morbidity statistics what might in all probability be the incidence of heart disease in the community, concerns the distribution of the cases affected both according to *age* and according to *etiological* groups. To make an analysis according to age commends itself especially on account of what has already been learned of the rising tide of chronic diseases which are naturally associated with older age groups. (See Table IV and Fig. 9.)

If, for instance, the age forty is taken as a dividing point it appears that in syphilis 19.7 per cent of the cases are under forty years of age, whereas 80.1 are over. And if 957 cases of degenerative heart disease are considered, again with age forty as the dividing point, 6.0 per cent occur before the age of forty and 93.2 above. Syphilis and the degenerative diseases combined occur above forty to the extent of

TABLE IV.

Distribution of Muscular and Valvular Diseases of the Heart in Relation to Age Forty.

Morbidity	No.	Per cent before age 40	Per cent after age 40	
Syphilis	91	13.6	86.4	Romberg[30]
Syphilis		16.3	83.4	Wyckoff and Lingg[31]
Syphilis		29.3	70.7	Stokes[32]
		Av. 19.7	80.1	
Arteriosclerosis	234	0.4	99.5	Wyckoff and Lingg[31]
Muscular degeneration	723	11.6	87.0	Romberg[30]
	Total 957	Av. 6.0	Av. 93.2	
Valvular heart disease	399	59.2	40.8	Romberg[30]
Valvular heart disease	670	73.68	27.32	Romberg[30]
Valvular heart disease	210	63.55	37.25	Schnitt[33]
Rheumatic heart disease	449	83.2	16.8	Wyckoff and Lingg[31]
Rheumatic heart disease	201	68.5	31.5	Fatianoff[34]
Rheumatic heart disease		65.3	34.7	Leuch[35]
Rheumatic heart disease	494	95.6	3.8	Church[2]
Rheumatic heart disease and chorea	191	94.3	5.7	Mackie[36]
	Total 2614	Av. 75.41	Av. 24.73	
Mortality				
Organic disease of heart		14.6	85.4	International List of Causes of Death No. 90

86.6 per cent. If, however, instead of muscular or degenerative diseases we study 2614 cases of valvular disease, 75.41 per cent occur under age forty and 24.73 per cent above this age. From the point of view of mortality rates, age forty seems likewise to be a useful point of division, for here it will be remembered 26 per 100,000 or 14.6 per cent of the total cases die before age forty in a total death rate of 178, a ratio of 1 to 6.85. There appears now to be a difficulty like that which was encountered before, for these facts seem to indi-
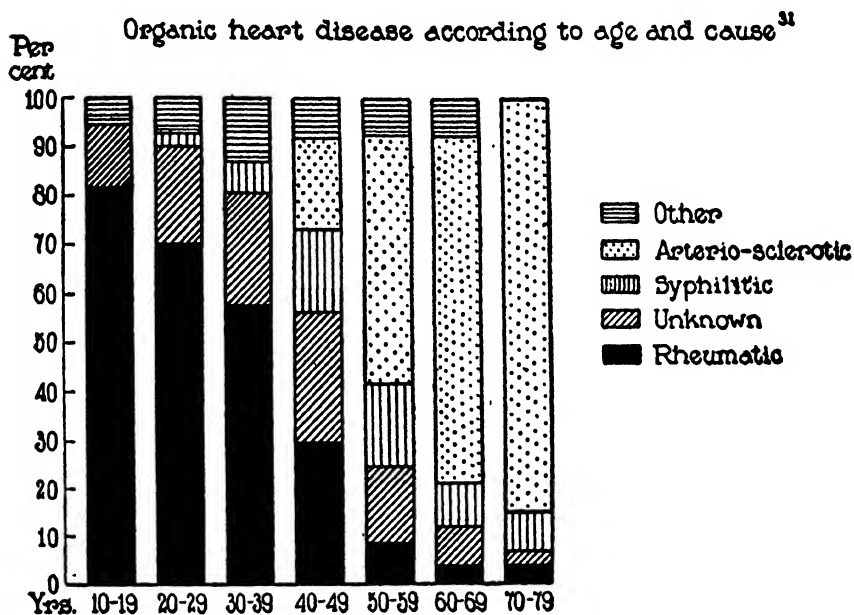
FIG. 9.

cate that 33.70 per cent (the average 19.7 + 6.0 + 75.41) of clinic patients both in Germany, the United States, and England are less than forty years old, whereas the mortality rate below forty years is only 15 per cent. The reason for this difference as before is difficult to understand. Wyckoff and Lingg have attempted to explain it; I shall return to this point later.

The age distribution then appears to give a clue to the nature of diseases with which we must deal. It appears that the valvular

diseases are predominantly the diseases which occur before the age of forty, the muscular diseases and syphilis afterward.

There is another way of approaching this matter of the division at the age of forty. It is well known that infection from rheumatic fever has its point of maximum incidence before age ten. It is true that this age is variously given, being placed at age six and one-half (Ingerman and Wilson[27]) and at age seven (Miller-Poynton, quoted by Ingerman and Wilson[27]). But that the turn of the first decade of life is the important one from the point of view of onset is seen also by the sharp rise in incidence that takes place between entrants and leavers in British schools.

Infection due to rheumatic fever occurs, of course, at other age groups, but it is here at a maximum. Church[2] in a study of 943 cases of rheumatic fever gives the age incidence under ten as 13.99 per cent; under twenty, as 57.57 per cent; and under thirty as 83.23 per cent. It is difficult to assign a figure to represent accurately the duration between onset and affection. There seems to be, relatively speaking, a small amount of data on the subject. But Coombs[37] states it as a fact that 66 per cent of cases of heart disease following on rheumatic fever occur between the ages of five and fifteen. Thayer[38] gives the same percentage below twenty. Mackie's[36] cases below twenty were 55 per cent. St. Lawrence[39] cites the history of seven cases in which rheumatic fever preceded the detection of valvular disease at intervals ranging from 13 to 72 months. The figures for the seven cases are 13, 26, 41, 45, 53 and 72 months; the average being 41.6 months. In the seventh case heart disease was present before the appearance of the more usual rheumatic symptoms.

Among 25 cases of mitral stenosis, Mackie reports that the lesion was developed in twenty-four months in 22, but that it required five and one-half, six and one-half and five and two-thirds (chorea) in 3 additional cases. It may take then one to six years for the cardiac lesion to form. Meanwhile manifestations of rheumatic disease may be quite absent. Next, the length of time before heart failure begins, Romberg[30] gives as approximately seven years, in 102 cases of valvular heart disease in his Leipzig clinic. The stage of heart failure itself may on the average last, as he shows also in a study of 172 cases in Leipzig clinic, four and eight-tenths years. If now these three

periods are summed up,—(1) the period from the beginning of the infection to the establishment of the chronic valvular lesion, say one to eight years, the average about four years; then (2) from this point to the onset of heart failure, say about seven years; and finally (3) from this point to death, say about four years, the total period is somewhere in the neighborhood of fifteen years. On the assumption that Church's figures and those of Ingerman and Wilson[27] and Miller and Poynton are correct, and that the ages five to fifteen embrace the usual period for infection from rheumatic fever, the total course of the disease will have been run by the ages twenty to thirty years. Or if the higher figures in Church's table are to be considered, namely, those which assign 25 per cent of cases of rheumatic fever to the third decade, the twenty to thirty years age group, the disease will have run its course between the ages of thirty-five and forty-five. This calculation again permits the selection of age forty as an important point in a consideration of chronic heart disease for it has now been shown that this age is significant in the natural history of rheumatic fever, that it is moreover important in morbidity and mortality statistics.

In view of the great significance that rheumatic fever has in the early ages of life it becomes important to obtain some estimate of the number of cases in a population which suffers from this disease. Reference has already been made to Swift's[29] statement that there are 175,000 cases in the United States. To obtain data for this is difficult for rheumatic fever has rarely been a notifiable disease. (See Table V.)

There was a period, however, from 1891 to 1895 when the disease was notifiable in Norway. At that time it was found in a population of 2,020,840 (1900 Census) that among 1,302,298 (64.4 per cent) above the age of fifteen, 13,052 cases developed. The male population was 604,257, the attack rate 2.07 and the cases of rheumatism 6281. The female population was 698,041, the attack rate 1.9, and the cases 6771. The average attack rate equals 1.98. In the Practitioner's Inquiry carried out under the Ministry of Health[9] in a certain selected portion of England and Wales, 90,891 persons come on the panel of a population of 12,633,000. Among these 51 males and 58 females developed the disease. The attack rates were 0.88 for males and 1.76 for

females. For the age period sixteen to twenty-four, approximately like that in the Norwegian data, the attack rate was 1.995. There are figures for the city of Ulm during the years 1883 to 1900 which give attack rates for the ages ten to twenty as 1.93.

Swift[29] on the basis of mortality data for 1916 in the United States Registration Area says that the combined figures for rheumatic infection show a rate of 6.7 per 100,000. If as he believes the

TABLE V.

*Incidence of Rheumatic Fever.**

Norwegian, English and Welsh Practitioners, City of Ulm.

	Year	Age	Population		Attack rates per 1000		Cases	
			Males	Females	Males	Females	Males	Females
Norway	1891–1895**	15+	604257	698041	2.07	1.9	6281	6771
			Total 1,302,298***				Total 13,052†	
Practitioners	1922	16–24			1.995			
Ulm	1883–1900	10–20			1.93			
Practitioners	1922	All	57998	32893	0.88	1.76‡	5!	58
			Total 90.891††				Total 109	
Ulm§	1906	All			1.75	1.24		

  * Compiled from pp. 64–65 Reports on Public Health and Medical Subjects. No. 23. Ministry of Health, 1924.

  ** Years of compulsory notification.

  *** 64.4 per cent of total population of 2,020,840 in 1900 Census.

  † Age 15+ was 81.7 per cent of total notified (15978).

  †† Total population at risk 12,633,000.

  ‡ Females equal twice the males. Half the females at susceptible age, 16–24.

  § F. Prinzing, Handbook Medical Statistics, 1906, p. 340.

death rate represents 4 per cent of the cases affected there should be in the United States between 150,000 and 175,000 cases of rheumatic fever. On the same basis, the death rate being 6.7 per 100,000 and representing 4 per cent of the cases, the attack rate in the United States equals 1.67 per 1,000. It will be seen then that a comparison of the attack rates of the Norwegian, Ulm, and the Practitioner's Inquiry presents a certain degree of correspondence; the Norwegian

figure for both sexes being 1.98 (all ages above fifteen), the British figure being 1.32, the Ulm figure being 1.45, and Swift's 1.67.

It is not my purpose to discuss all the factors which make for the occurrence of rheumatic fever. The problem of possible bacterial agents has, as you know, engaged the attention of investigators for many years. I need go into no further detail than to recall the fact that there is no general agreement on which microorganism is the efficient cause nor indeed whether the cause is a microorganism at all.

The relation of damp and wet and cold is also often mentioned, especially in the older literature. There are two aspects of this phase of the problem, seasonal and geographical. It has been explored by many older writers, reviewed carefully by Pribram[40] and more recently by Newsholme,[41] Greenwood and Thompson,[6] Young,[42,43] the Practitioner's Inquiry,[9] and Miller.[11] Pribram, Newsholme, and Greenwood and Thompson have opposed the idea of a relation of rainfall to the occurrence of rheumatic fever; Garrod on the whole favored it as did Young, the Practitioner's Inquiry, and Miller. Opinion then is still divided. Miller reports that of 196 cases, 122 or 62.2 per cent occurred in damp rooms. He presents data to show that among persons who live in them or in basements or in ground floor rooms without cellars, the liability to the disease increases. The Practitioner's Inquiry[9] concludes that dampness has a higher correlation with acute rheumatism than with any other rheumatic disease. It was also shown that if the attack rates from rheumatic fever and rheumatism of the heart for all England and Wales were placed at 100, the attack rates for Lancashire may be placed at 159, the highest in England. Norwich, in East Anglia, is on the contrary exceptionally free from rheumatic diseases. Lancashire situated in the Northwest, has relatively the higher rainfall.

Some day perhaps we may recall in this connection Pettenkoffer's idea that the height of the ground water in Munich was related to the occurrence of typhoid fever.

A discussion of rheumatic fever is not complete without reference to the possibility of its infectious nature. Church[2] quotes Friedlander as having seen 12 cases in the same house in Leipzig in three years. He also quotes Edelfson as having counted 728 cases in 492 houses.

More recently a number of new reports have been published on its

TABLE VI.

Rheumatic Diseases.

Familial and Hereditary Aspects.

Familial	Disease	Number patients	Number studied Families	Number studied Persons	No. members in same family affected	Total families affected No.	Total families affected %	Affected persons discovered No.	Affected persons discovered %	Persons unaware of disease No.	Persons unaware of disease %	Remarks
St. Lawrence[45]	Heart disease	100	100	480	2 or more	29	29	38	8.0*	27	71	Usual 1–2%*
	Acute rheumatic fever		100	480	2 or more	24	24	49	10.0*			Usual 1%*
	Chorea		100	480	2 or more	3	3	8	1.6			
Ingerman and Wilson[37]	Rheumatism	185				74 Parents 51 B & S†	40 28					
Miller[21] (from Coates and Thomas) (from Morrison)	Rheumatic infections	44 32 54				21 12 26						
Coombs[37]							50					
Faulkner and White[44]	Rheumatic affections	200	200	1235*	2 or more	71	35.5	108	8.79			642 examined*
	Healthy controls	75	75	474*	2 or more	12	16.0	13	2.95			366 examined*

Hereditary	No. patients	Number	Per cent	Parent affected Fathers	Parent affected Mothers	Parent affected Both	Siblings affected Per cent
Faulkner and White[44]		332	8.9				8.66
Report 23[9] Males			24.0	13	21	8	
Females			43.0	7	15	2	

* Compare with statement on same line under remarks.   † B & S—Brothers and sisters.

familial and hereditary aspects.   (See Table VI.)   Coombs[37] gives 50 per cent as the incidence in families, in which there were cases in addition to his patients.   St. Lawrence[45] in a hundred families gives the figure as 29 per cent for rheumatic heart disease; Miller[22] quotes Coates and Thomas as giving 47 and 37 per cent affected, in two series, and Morrison, as giving 48 per cent; Faulkner and White[46] give the rate as 35.5 per cent.   In the control families of healthy individuals, the per cent falls to 16.   In rheumatic families, if I may so call them, there were 8 to 10 per cent of exposed persons infected as against 1 to 2 per cent in the population at large, and in the families of healthy controls only 2.95 per cent.   These figures are striking enough to stimulate more searching inquiry in this direction.   It is a phase of the problem which was not without interest in the study of tuberculosis, and is now the subject of research in lobar pneumonia.

It is not surprising that with a disease incidence as large as that in rheumatic fever much thought has been expended on the possibility of remedying this situation.   From the point of view of the public health to institute a serious effort in this direction is eminently desirable.   On account of the frequent association of other affections, more especially that of the tonsils, with this condition attempts have been made to modify the incidence and course of rheumatic fever by attention to this source of infection.   Unfortunately much information does not yet exist on the subject.

There are, however, several series of cases, beginning in the year 1908, in which reports have been made on the effect of operation on the tonsils.   (See Table VII.)   It is perhaps important to distinguish the cases in which removal of the tonsils was complete, that is to say the cases in which the tonsils were completely enucleated and those in which the enucleation was incomplete.   There were 391 cases observed before and after operation.   The rheumatic manifestations for which the operations were performed recurred in 49.6 per cent of these.   This figure is low rather than high, for recurrence involves consideration of the period of time after operation during which observations were carried on, as it has been found that the percentage rises with time.   This point is especially important for in several instances enumerated in this table the duration of observation has been short, a matter of months rather than of years.   In connection with these 391 cases, 2,400

TABLE VII.
Tonsillectomy.
Its Effect on the Occurrence of Rheumatic Manifestations.

Date	Author	No. cases total	With Tonsillectomy		No recurrence		With recurrence		Without Tonsillectomy		No development of manifestations		With development of manifestations		Remarks
			No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	
Complete Tonsillectomy															
1908	Rosenheim[49]	7	10		10	100	0	0							
1911	Archibald[50]	21	7		5	71.4	2	26.8							
1915	Young[51]		21		6	28.6	15	71.4							
1917	Crewe, Watkins and Rotholz[52]	49	49		28	56.3	21	43.7							
1920	St. Lawrence[38]	85	85		54	63.5	31	36.5							
1923	Hunt and Osman[47]	144	66		31	47	35	53	78		45	58	33	42	All cases
		(99)	(50)				(23)	(46)	(46)				(15)	(33)	After first attack
		(48)	(16)				(12)	(75)	(32)				(18)	(56)	After later attacks
1924	Ingerman and Wilson[37]	167	70		17	24	53	76	97		19	19.6	78	80.4	
1924	Dulaney[53]		22		22	100.0	0	0							
1926	R. Miller[55]	133	45		18	40	27	60	(83)						
1926	R. Miller (A. P. Thompson)[48]	16	6		6		10	62.5							
1926	Mackie[36]	299	80		46	57.5	34	42.5	207		82	39.7	125	60.3	After first attack
			12		0	0									Before first attack
Totals			483		255	52.8	228	47.2	382		146	38.2	236	61.8	
1926	Kaiser[33]	2400	1200						1200				128		Rheumatism
Partial Tonsillectomy															
1921	St. Lawrence[38]	9	9		1		8								
1924	Ingerman and Wilson[37]	18	18		4		14								
Totals:		27	27		5	18.0	22	82.0							

cases studied by Kaiser[23] should be mentioned. It is unfortunate that Kaiser has not yet published analyses of his large number of carefully studied cases in such detail as to make it possible to enter the results in a table such as this. In order to be able to do this, it is of course necessary to be able to follow the course of the disease in each individual. Few investigators have as yet appreciated the importance of this method of presenting data, especially outside the United States. In the group in which enucleation was incomplete there were 27, among these 22 (82 per cent) recurred.

DEATH RATE PER 100,000.
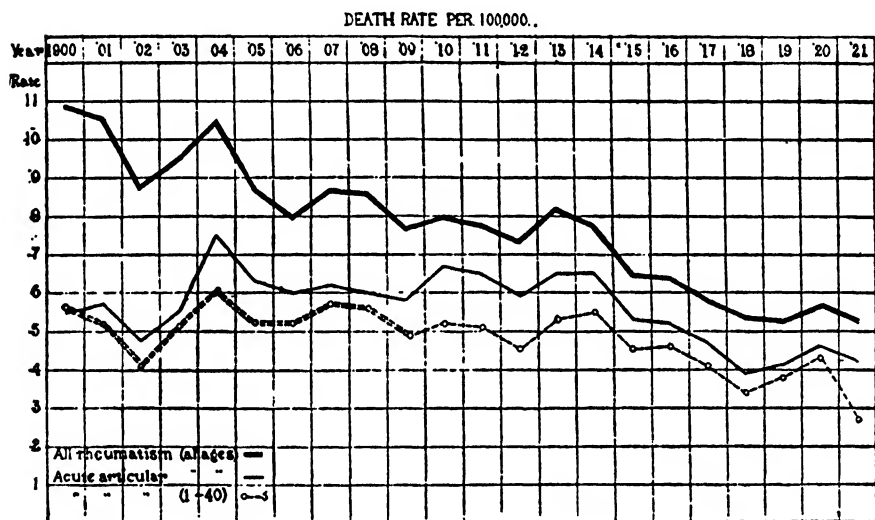
FIG. 10.—Death rate from all rheumatism, and from acute rheumatic fever in New York State, 1900–1921.[54]

There have been reported several groups of cases in which no operations on the tonsils were performed. These serve as controls. Of well studied cases, 175 may be mentioned, among which rheumatic manifestations developed in 111 or 64 per cent. The largest series of cases studied from this point of view is Kaiser's.[23] Twelve hundred cases were observed. Of choreic manifestations there occurred 7, of rheumatic 128, and of cardiac 52. But among the cases operated upon there were 8 instances of choreic, 129 of rheumatic and only 44 of cardiac manifestations; that is to say there were more rheumatic and

choreic manifestations in those operated upon than in the so-called controls. It is necessary to speak of manifestations rather than of cases for the number of cases which the manifestations involved is not

DEATH RATE PER 100,000 (AGES 1-40 INCLUSIVE)
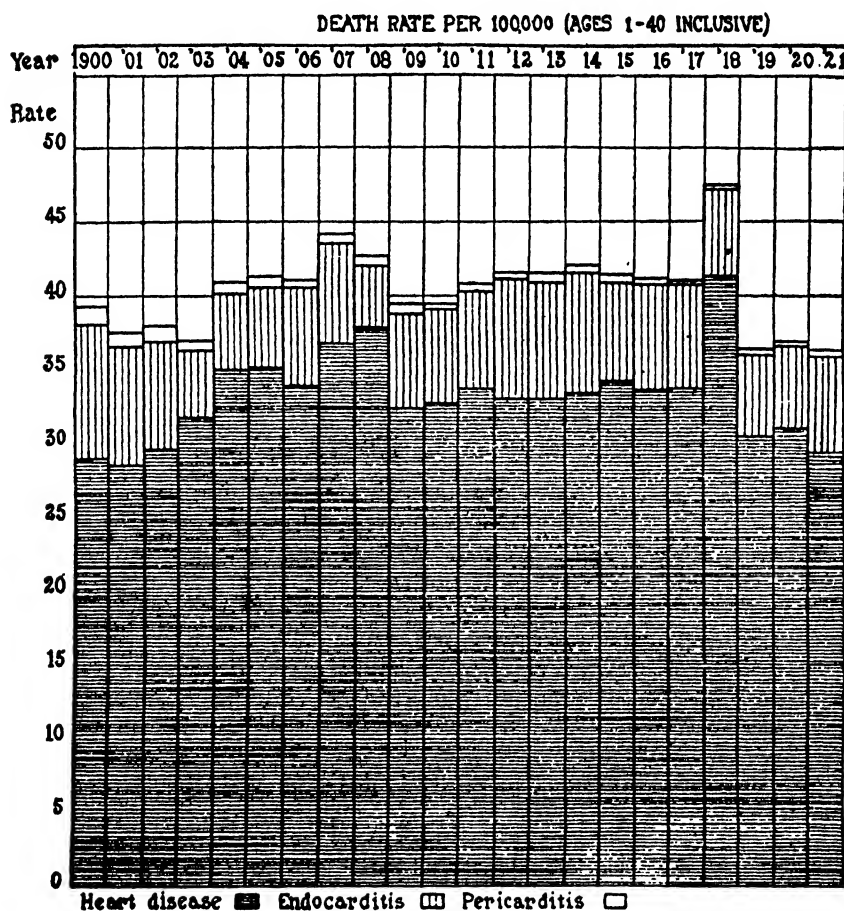
FIG. 11.—Specific death rates (ages one to forty years inclusive) for heart disease, endocarditis and pericarditis in New York State, 1900–1921.[54]

given. The most signal advantage of the operation seems to have been in the cases of tonsillitis for here among the operated there were only 64 cases as against 586 in the nonoperated. Unfortunately the effect of operation on the matter of recurrence, the essential object of study,

is not deducible from Kaiser's report.   On the whole then, it cannot be said that the results of this therapeutic measure are satisfactory, recurrence taking place in 50 per cent of the cases.   When series of cases reported by individuals are considered, attention must be paid to St. Lawrence's view[39] that especially in cases in which chorea is not a factor the performance of tonsillectomy is an extremely important measure.   To discuss each series of cases is important but impossible here.   Miss Lingg, my associate in the Heart Committee in New York, is at present engaged in making a complete analysis.   Interpretation is difficult, for not only as has already been said must time of observation be taken into consideration but also the time of operation in relation to the onset of rheumatic disease and especially the relation to first or second attacks.   Although from Kaiser's observations tonsillectomy seems to make no difference from important points of view, this method of therapeutics must nevertheless be carefully pursued precisely for the reason stated.   It is urgently desired that observers record their cases in such a way as to make it possible to deduce what has actually been the course of events in their patients.

Of other means of preventing and relieving rheumatic fever, only a few words need be said.   The use of drugs, as effective agents of cure, must unfortunately be dismissed.   Careful investigators are unanimous in their opinion that no hope lies in the direction of the use of those which have so far been proposed.

But that, as in the case of tuberculosis, something may be accomplished by prolonged and complete rest, the reports of Swift[54] more recently published indicate clearly.   Coleman[55] has insisted on this point and has illustrated his contention with telling case reports.   Experiences like these emphasize the fact that in order to duplicate them, facilities in the way of beds in hospitals and in convalescent institutions properly supervised are requisite.   They tend also to lend point to the general observation that treatment of patients in private, in the economically more self-sufficient walks of life, is attended by prolongation of activity and of life.   That the failure to provide such facilities possibly shortens usefulness and life will appear in considering the curves of Wyckoff and Lingg.

Aside from the analysis which has already been made showing the very partial success of tonsillectomy on the course of rheumatic infec-

tion, a study of the death rate curve in New York State made by Swift[54] shows that no substantial change has occurred in ages below forty either in endocarditis, in pericarditis or in organic heart disease. (See Figs. 10 and 11.) His curves for rheumatism and for acute rheumatic fever show a persistent fall and encourage one in the pursuit of the therapeutic measures which have been described.

If we return now to consider the etiological groups which have contributed to the total of deaths after age forty, the largest number of cases are due to senescence. A certain number are found to be sequels of syphilitic infection. There are included, also, a number of cases acquired at this age, of valvular heart disease, and perhaps of muscular disease too, due to rheumatism and to a lesser extent to other infectious diseases, and a certain number due to infection acquired before this period. Arrival at old age brings with it certain disadvantages. It not only ushers in the natural probability of death by a normal cardiac mechanism, but on account of the inherent defects of the system in advancing years, exposes individuals to injury, as Dublin[56] suggests, to which they were earlier not prone. Exactly which these might be from the point of view of the circulatory system is not yet quite clear. But if this system were so affected, a disease of old age, rather than old age itself, will have occurred; cases of this nature would perforce swell the cardiac death rate. But the overwhelming majority of cases must be due, if the considerations which have been urged are correct, to the cause just mentioned. It has already been shown that five-sixths of the deaths from heart disease occur after age forty. If these were due in large measure to muscular and vascular rather than to valvular diseases one would expect if the muscular diseases were separated to find a greater percentage of cases of this type after age forty. That this is actually a fact has already been shown (Table IV). Of 957 cases, 93.2 per cent were forty and over.

The duration of muscular disease of the heart has been studied by Romberg[30] in 176 cases in the Leipzig clinic. It was found there that the interval between the time when the first attack was experienced and when the heart began to fail averaged fourteen and one-half months. The time between the beginning of heart failure and death averaged five and one-half months, ranging from a few days to two to three years. On this basis the total duration is put down as one

and one-half to two years, ranging from a few days to twenty years. Cases were known to last as long as ten to fourteen and even seventeen to twenty-three years.

That cases of syphilis also fall into this group, appears from the fact that 80.1 per cent occur after forty (Table IV). What the number of cases of syphilis is in the United States and what per cent of these develop cardiovascular disease is very difficult to ascertain. I can find no adequate estimate of either group. In the Registration Area for 1920, the death rate for syphilis is placed at 9.1 per 100,000 population or 9,100 deaths. Stadler[57] puts the occurrence of syphilitic aortitis among syphilitic autopsies in the Leipzig Pathological Laboratory at 82 per cent. Hubert[58] gives the ratio of aortitis to visceral syphilis at 70 per cent. At this rate there would be 7,362 cases of syphilitic aortitis in the country, a rate of 7.36 per 100,000 population. In comparison with this figure, the incidence of syphilitic aortitis among general autopsies is very high. Gruber[59] (p. 96) gives the figure as 4 per cent (4,000 per 100,000 population), Lamb[60] as 4.58 per cent, Oberndorfer[61] as 6.89 per cent. In a revision of the Registrar General's Mortality Statistics by Osler,[62] apoplexy, aneurysm and aortitis, organic diseases of the heart, and diseases of the arteries, all told caused 92,564 deaths; of these 12,000, 12.9 per cent, were due to syphilis. In Bellevue Hospital[53] of all admissions in 1915, 30.5 per cent are said to be syphilitic. In certain industries in New York those infected are 22 per cent.[63] Elsewhere in the country the rate falls as low as 7 per cent.[63] Since the introduction of the Wassermann reaction, the number of cases in general medical clinics diagnosticated as syphilis has increased. In 1896 (Mengel,[54] Leipzig) it was said to be rare; in 1919 (Adlmühler,[65] Munich) it was put at 18 per cent and in 1924 (Wyckoff and Lingg,[31] New York) at 8.6 per cent. Steinfield, Pfahler and Klauder[66] found it only in 11 per cent of syphilitic males, a low figure in a group which would generally be regarded as favorable for yielding a high figure.

The matter may be summed up in the following way:

Mortality
Syphilitic deaths.   Registration Area.   U. S.   9.1 per 100,000
Syphilitic cardiac deaths are of all cardiovascular deaths
                              Registrar General's Report   12.9 %

| Of syphilitic autopsies | Syphilitic heart disease represents | 82.0 % |
| Of general autopsies | Syphilitic heart disease represents | 4–6.85% |

Morbidity

Syphilis	General morbidity	7–30%
"	in general hospitals (Bellevue, N. Y. C.)	30%
"	in industries N. Y. C.	22%
"	elsewhere in the United States as low as	7%
"	in syphilitic cardiovascular cases equal	11%
General cardiovascular disease-syphilitic cardiovascular cases equal		8–18%

A low estimate places the number of persons in the country infected with syphilis at about 1,000,000. In choosing this per cent I am following the example of a Royal commission[32] (p. 39). If 11 per cent represents the proportion that develops cardiovascular diseases in a syphilis clinic the number may be placed very roughly at 110,000 cases. And if 8 per cent (Wyckoff and Lingg) is the proportion of syphilitic heart disease in a general clinic, the number of syphilitic cardiovascular cases would be 120,000, on the assumption that the total number of cases of heart disease in the country is between 1,000,000 and 2,000,000 cases. This agreement is fair but it is based on the roughest of estimates and is entitled to no great confidence.

But it is more important now to try to find the age incidence than the actual number of cases of syphilitic heart disease. (See Fig. 12.)

Stokes[32] has arranged 190 cases in the curve to the left in five year age groups. He has not published a scale, but by measurement I find that 10.9 per cent (24 cases) were below thirty-five years and 29.3 per cent (55 cases) under forty years. A large majority then is above forty. In the middle curve he shows that few cases relatively speaking have an incubation period less than ten years; that in most cases this period lasts from eleven to thirty years, the greatest number, sixteen to twenty years. If syphilis is acquired in the second and third decades and if the time before the development of symptoms is usually about twenty years, the appearance of these would generally be expected about age forty. Mackenzie (1908) mentions a case (quoted by Romberg[30]) in which cardiovascular phenomena were discovered seven weeks after infection; but it is unusual to find them before two years.

If now the distribution of heart disease according to etiology and age groups is undertaken it is found that a curve expressing the sum

Cardiovascular syphilis
Age incidence - Duration before onset of symptoms[32]
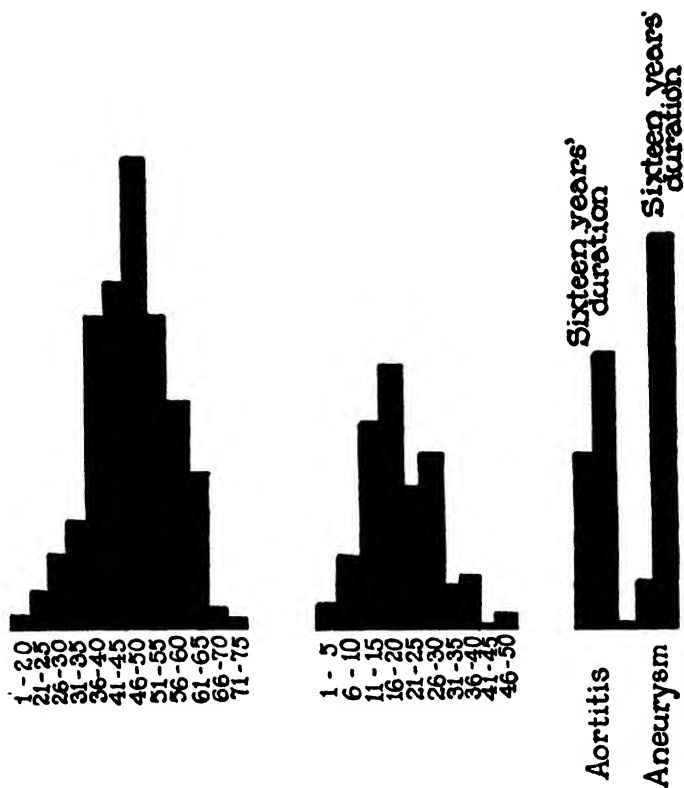
FIG. 12.—In the figure to the left, the relative incidence of cardiovascular syphilis for various age groups is given. In the middle figure is indicated the relative number of cases in which the duration between infection and onset of symptoms equalled the number of years given below. To the right, the division between the long and short columns in each case is sixteen years. Before sixteen years more cases of aortitis (and of aortic valvular insufficiency) are detected than of aneurysm. Aortic insufficiency has a shorter "incubation" period than aneurysm, apparent rather than real, because on account of its conspicuous physical signs it can be detected earlier than aneurysm. (From J. H. Stokes,[32]).

of the cases of syphilis and of arteriosclerosis added together which occur both in public and private practice falls far below the curve of morbidity which one should expect and which would run parallel with the mortality curve for these age periods. This statement applies to data collected not only in this country but also abroad. Wyckoff and Lingg have studied this problem in some detail and from their study follows a consideration of value for interpreting the data which come from the clinics for public patients, and perhaps also, but to a less extent, in private patients. These are the curves to which I have already referred. If it is true that the natural history of heart disease whether from rheumatism, from syphilis or from old age follows a usual course, then the curve of morbidity should follow the curve of mortality in some usual fashion. The curve of mortality must, within limits of course, be considered as the norm.

In these curves (Fig. 13) taken from Wyckoff and Lingg the mortality of New York is given in the uppermost. Obviously the curves for ward patients and the curves for private patients do in some measure resemble the mortality curve, but the curve for public ambulatory patients and of course the curve of all ambulatory patients public and private together fails signally to resemble it. In the first place the apex of the second mode of the public ambulatory patient curve is found to be twenty years before the apex of the mortality curve and ten years before that of the private patient curve. The public patient curve moreover falls abruptly ten years earlier than that of the private patient curve. Wyckoff and Lingg think furthermore that the first part of this curve is higher than it should be because the numbers were augmented by the inclusion of patients brought to the Bellevue clinic from outside the Bellevue district, from the schools and from social service sources not connected with the hospital. There results in consequence a distortion of the curve which might occur elsewhere also. Why the second portion of this curve, that after age forty, fails to follow the mortality curve is not clear. Indeed the matter is difficult to understand, but Wyckoff and Lingg suggest the following explanation. People come to the clinics in early years because rheumatism is a dramatic disease and entails great suffering. The heart disease which results from it occurs when people are quite young and when the hope of a useful life is still strong. Accordingly they seek relief.

Organic heart disease
Curves of morbidity and mortality[34]
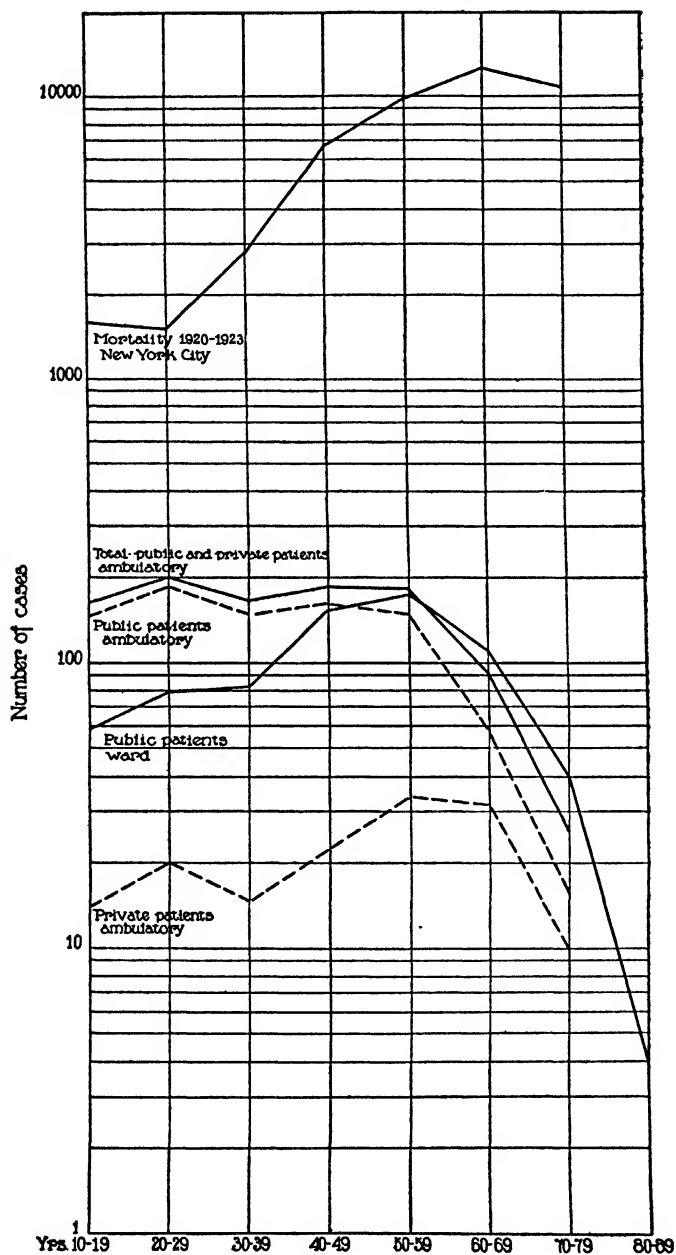
FIG. 13.

The clinic curve is consequently high.   Heart disease in the older age groups comes on insidiously.   The meaning of the symptoms is misunderstood.   The outlook in life is no longer full of promise.   Economic pressure has been relieved either on account of savings or because patients are supported by children or otherwise.   The situation is one which in any case is anticipated as part and parcel of the gradual process of decay.   Patients do not come to the clinic; they do not seek advice; they accept the inevitable.   The clinic curve falls, precisely at the moment when one would expect it to be high.   If this description represents the situation in public practice the curve for private practice ought to differ from it and in point of fact it seems to do so although it does not follow the mortality curve as closely as it might. But the experience here is small and it is not beyond possibility that the argument which applies in public practice applies also in private. This is a subject which requires further and more detailed study.   But in these considerations there is included a warning.   Data obtained especially from public practice require scrutiny with the view to making certain that the samples offered as illustrative of the course of disease actually are so.

With the bearing of race and of sex on the incidence of heart disease I propose not to deal.   Their influence on the phase of the problem I am discussing is not sufficiently clear.   That girls at the age of puberty to twenty, according to Osler, or to thirty-four, according to the Practitioner's Inquiry,[9] fall subject to rheumatic fever twice as often as boys, though contrary opinions are also expressed, and that heart disease due to syphilis is more common in men, is well known.

It appears also to be a fact that the duration of life in negroes subject to heart disease is a decade less than among the white population. The incidence of heart diseases among them seems to be greater everywhere in the Union, as is seen in the statistics of mortality when the State groups are studied; theirs exceeds the rate of the white population by 26 to 68 per 100,000 wherever their population is over 1,000,000. When it is small the rate rises as it must if the scale is per 100,000. The difference is assigned by Dublin[12] to the greater incidence of syphilis, malaria and typhoid fever.   Higher rates in earlier age groups may naturally be due also to decrease in natural longevity.

I have laid so much stress on age forty as a dividing point not only

because, as it appears, the majority of cases before this period have their origin in infectious diseases and especially in the rheumatic group, but because I wish to emphasize the fact that these are relatively speaking a small number, namely one-sixth, of the total; that in point of fact by far the greater number of deaths due to heart disease, namely, five-sixths, occur after this period. On the far side of age forty, a certain number are the sequels of syphilis. But presumably the larger number results from what is usually called degenerative disease; from arteriosclerosis. 1 wish to contend that these conditions are properly not diseases at all, but that they represent differentiation phenomena in the course of the ordinary growth process. Multicellular animals, and the argument need be suggested only, have if not a fixed, at least an anticipated longevity. From the point of view of expectancy years have been added to life. Unfortunately this fact has sometimes been interpreted as being a biological rather than an arithmetical phenomenon. There is sometimes confusion between the meaning of expectancy and of longevity. The duration of life has not been extended; on the contrary the attainment of its natural length has merely been rendered possible. How this may have come about at least in part has appeared in studying the relation between the declining death rate from infectious diseases and the rising one in chronic diseases (Figs. 3 and 4). But the conclusion that the increased number of deaths from heart disease has occurred in the older age groups exclusively results from the study of specific death rate curves. (Fig. 14.)*

All the heavy dotted curves marked C at the bottom of the figure that is to say curves of heart disease, one for each decade below age forty run, to all intents and purposes, a horizontal course. The curve for ages 0–4 on the contrary has fallen sharply. Into the cause for this I need not enter. All the light dotted curves in the midregion of the figure, one for each decade marked A B D, that is to say the curves representing infectious diseases below age forty, have likewise fallen. All the light solid lines marked A B D one for each decade over age forty have either fallen or remained horizontal, except for age ninety and this one too has fallen in the last ten years. That the death rate

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* I am indebted to Miss Alice Whittemore for the data in this and the next two curves.

from heart disease below age forty has fallen is apparent also in Curve C_2, Figs. 3 and 4. In what decades the rates from infectious diseases have been affected, the movement of the total rate of which is seen in Figs. 3 and 4, is shown in Fig. 14 in which age specific curves are exhibited. It appears on analysis then that the rise in the death rate from heart disease has occurred exclusively after age forty, as the double lined curves one for each decade marked C show, and the rise has been the higher the higher the age.

If the increase in deaths from heart disease has resulted from transferring deaths due to defects in the arterial system or from some other indefinite heading to the cardiac group, a fall in the curve for diseases of the arteries should have taken place. That this is not the case is seen in these curves. (Fig. 15.) And that the changes have taken place exclusively above age forty is apparent from this further set. (Fig. 16.)

A summary of the changes which have taken place in the past ten years is exhibited by the curves of Fig. 17.

In Fig. 17–A are age specific curves for 1910 and 1920, both for males and females.* With the exception of a single half decade there

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EXPLANATION OF FIG. 14.

Diseases grouped as follows:

A—(Infectious diseases)	Malaria	Other diseases of the
Typhoid fever	Cholera nostras	heart (including en-
Measles		docarditis)
Scarlet fever	B—Tuberculosis	Diseases of the arter-
Whooping cough	Pneumonia	ies
Diphtheria	Bronchopneumonia	Other diseases of the
Influenza		circulatory system
Mumps	C—(Circulatory diseases	D—(Acute cardiac dis-
Dysentery	exclusive of acute	eases)
Acute poliomyelitis	cardiac diseases)	Pericarditis
Meningococcal menin-	Apoplexy	Acute endocarditis
gitis	Angina pectoris	and myocarditis

States included: Connecticut, Indiana, Maine, Massachusetts, Michigan, New Hampshire, New Jersey, New York, Rhode Island, Vermont, and District of Columbia.

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* For the data for these curves I am indebted to Dr. William H. Davis, Chief Statistician for Vital Statistics, Department of Commerce, Bureau of Census, Washington.

Specific death rates, first ten registration states·D.C. for certain
groups of diseases 1870-1920
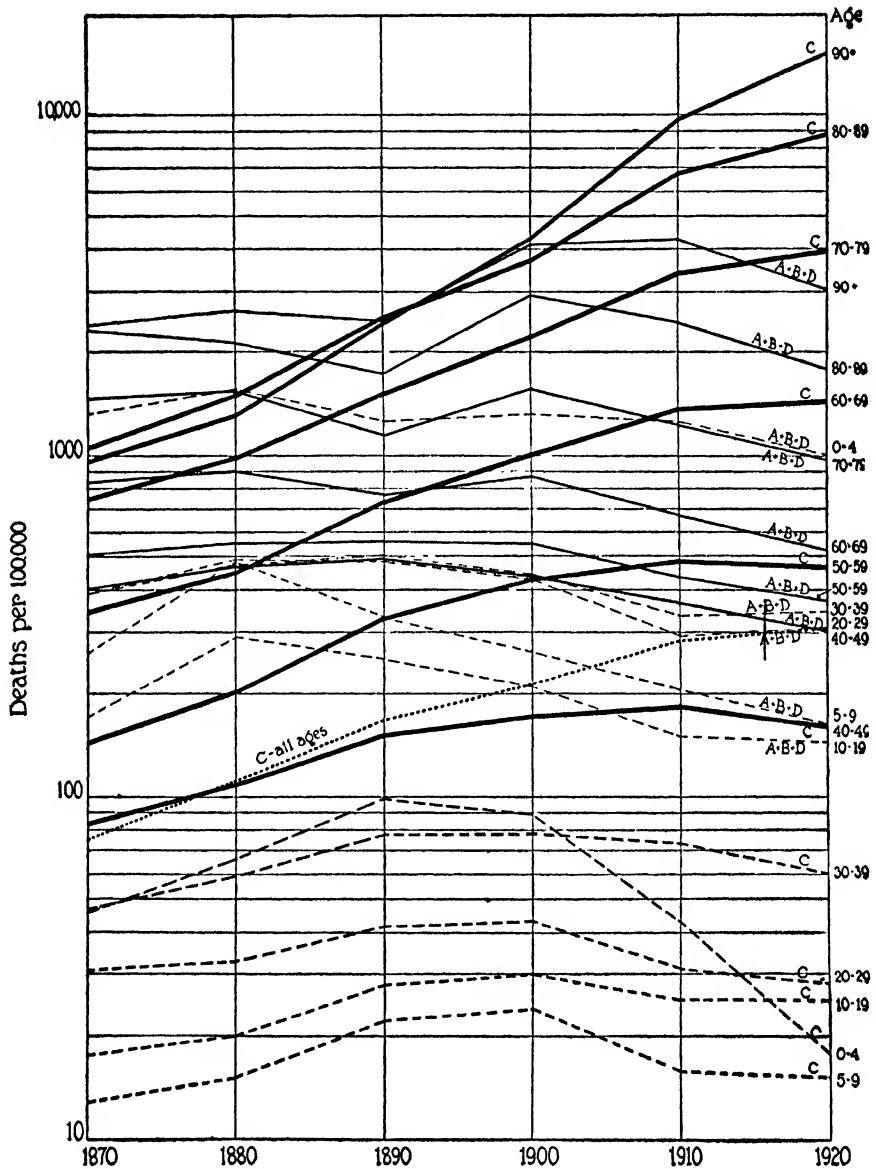
FIG. 14.

Specific death rates, first ten registration states +D.C. for diseases of the arteries 1870-1920

All ages

Age 40+

Age 0-39

Rate per 100,000

100

10

1

1870   1880   1890   1900   1910   1920
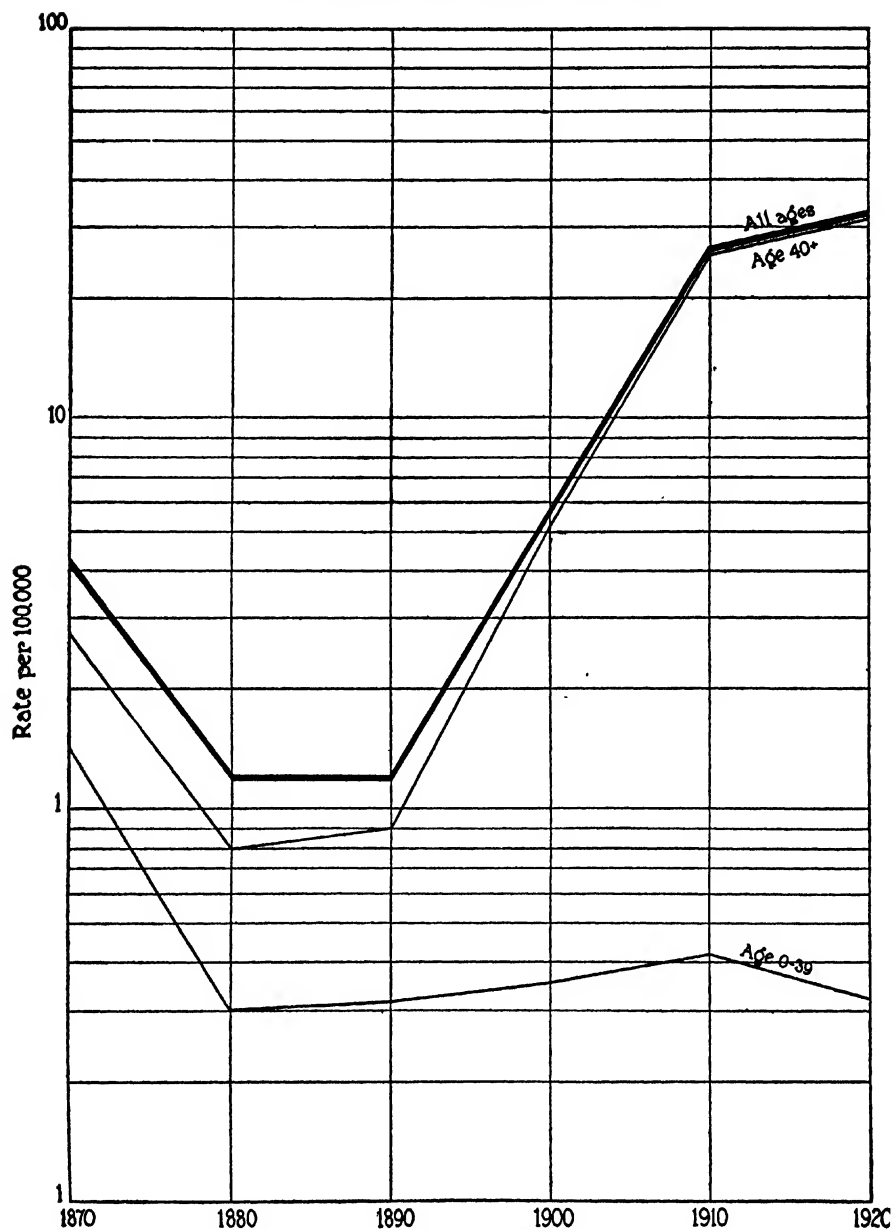
Fig. 15.

Specific death rates, first ten registration states plus D.C. for diseases of the arteries 1870-1920

Fig. 16.

387
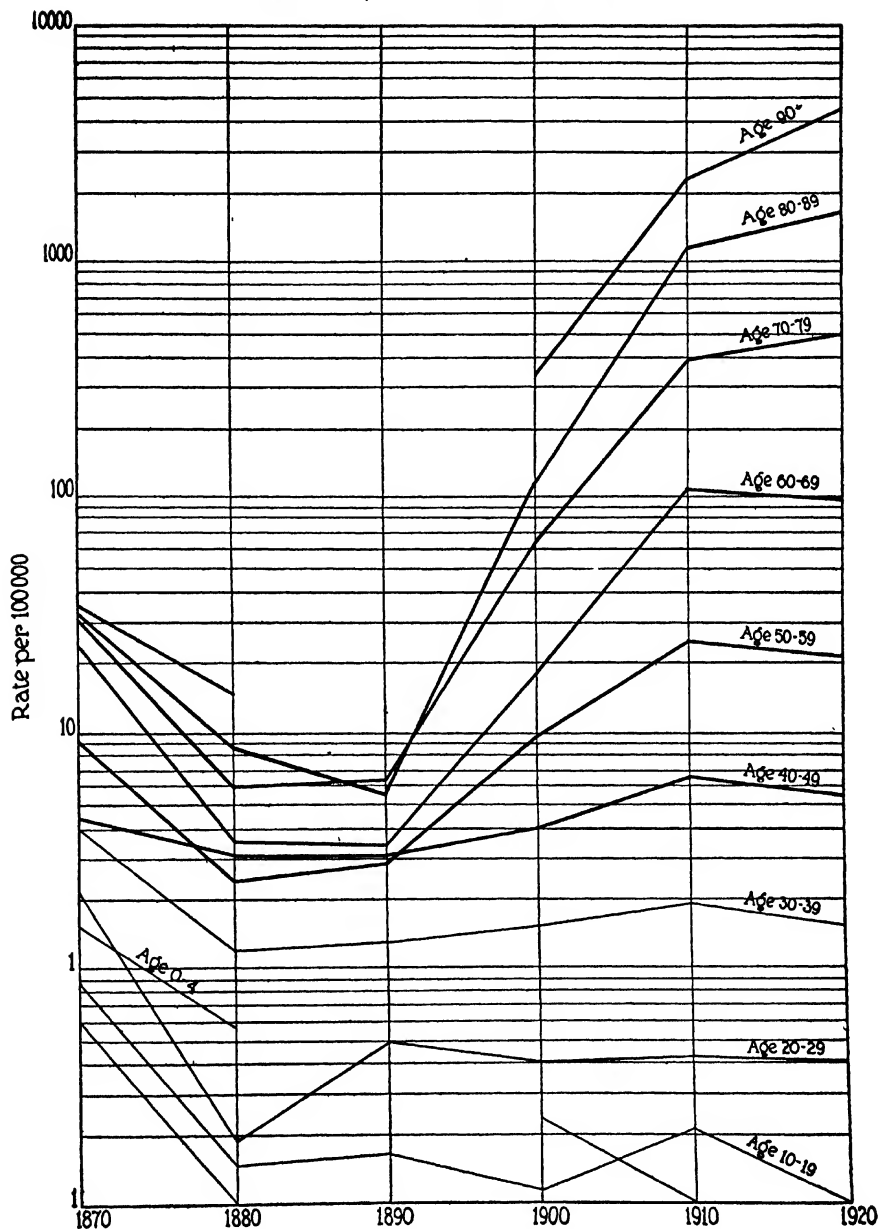
Death rate per 1000 living of each age and sex for Heart Disease in the U.S. Registration States, exclusive of N.Carolina in 1910 and 1920

Difference in deaths -304 +25 +66 +206 +76 -25 +54 +109 +62 +89 +608 +1166 +1873 │ +3221 +3239 +4232 +5679 +4797 +3152 +1230 +734 +25
Difference in rate -37 -02 -02 +.01 -.01 -.03 -.06 .13 -.16 -.17 -.10 -.10 │ +.07 +.87 +2.02 +5.73 +12.79 +24.09 +47.57 +87.28 +24.22

Average decrease for 1920 .09　　　　　　　　Average increase for 1920 17.12
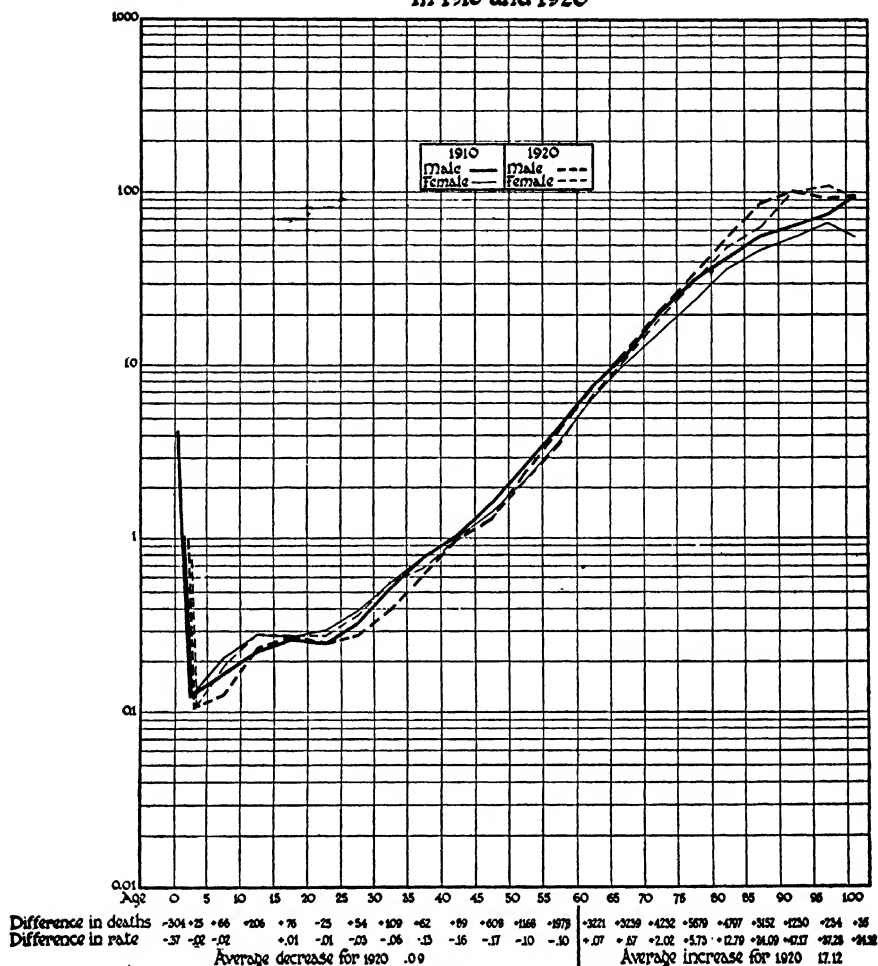
* This includes Pericarditis - Acute endocarditis - Organic diseases of the heart - Angina pectoris - Diseases of the arteries - Embolism and thrombosis - Diseases of the veins - Diseases of the lymphatic system - Hemorrhage; other diseases of the circulatory system: Nos. 77 to 85 of the International List

FIG. 17-A.

Death rate per 1000 population for Heart Disease in the U.S. Registration States, exclusive of N. Carolina in 1910 & 1920

Fig. 17-*B*.

389
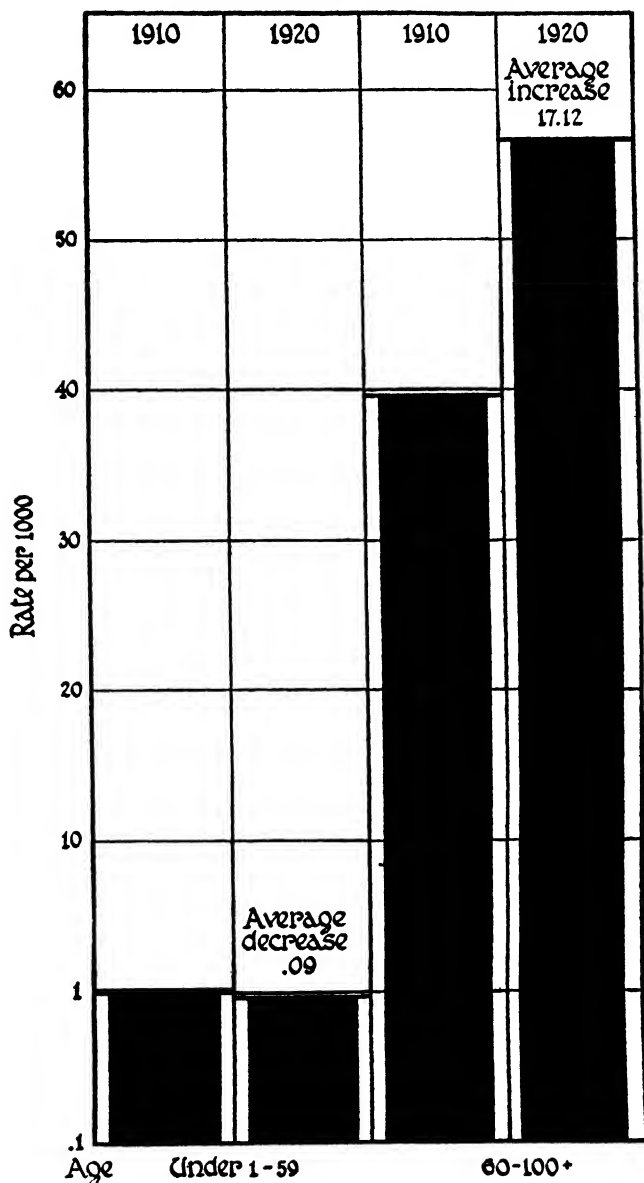
is a fall in rate for each decade before age sixty. The fall between ages forty to sixty concerns the year 1910 only; in earlier years there is a conspicuous rise as has already been shown. Above age sixty there is a uniform elevation of the 1920 curves above those of 1910.

In Fig. 17–B there is still another expression of the same phenomenon. Under age sixty, the rate fell 0.09, above age sixty, it rose 17.12.

Aside from the matter of treatment with drugs and by other means, of which I have already spoken in connection with rheumatic fever and syphilis, the large number of persons who. suffer from chronic heart disease places a charge on the public to see to it that.provision in appropriate institutions is made for their care. Public provision at least of two sorts is requisite; the hospital for what may be called the more acute or urgent phases of these diseases or, in the case of rheumatic fever, during the period of active infection in the febrile stage; and in the case of already established chronic disease, in the period when heart failure first appears. Later, for both types of case, further provision is desirable when the course of disease is less stormy and while reconstitution on a new level of activity is being accomplished and a new way of life learned. What has come to be known as the convalescent home now supplies the second need.

Unfortunately I do not possess data covering the hospitals in the whole country. But in New York, I have figures which Emerson[67] has published. He found in 1920 or 1921, that in ten of the larger New York hospitals 98,838 patients were admitted. Of these 4.6 per cent (4,522 cases) were cardiac. The average stay in hospital for each cardiac patient was fifty-two and two-tenths days. The per cent of all hospital days care given to cardiac patients was 9.35. Notice that cardiac admissions were 4.6 per cent, whereas cardiac patients took 9.35 per cent of the hospitals' attention. The cost of a cardiac patient in the general hospitalization studies averaged $257.27. He suggested that a one-hundred-bed convalescent cardiac hospital was needed. Dr. Emerson has in a letter explained to me the course of his reasoning. On account of its interest, I repeat his argument. Although heart patients represent only 4.58 per cent of all general hospital patients, they require, or at all events receive, twice as much care in proportion to their numbers, as other patients do, or 9.35 per cent of the total number

of days given in the hospital study. Assuming that they were still in need of further bed care, probably an additional twenty to forty days per patient, a total stay of forty to eighty days of hospitalization for each cardiac patient is estimated. Those of us who have had experience in hospitals know that not even this allowance suffices. They can in point of fact be better and more economically cared for

TABLE VIII.

Summary of National Directory of Convalescent Homes.[88]

States	Homes	Year round beds	Summer only	Total
Alabama	2	12	20	82
California	11	489	100	589
Connecticut	5	120	280	400
Georgia	1		50	50
Illinois	8	292		292
Kentucky	2		80	80
Louisiana	2	30	50	80
Massachusetts	24	476	228	704
Maryland	4	78	44	122
Michigan	1	135		135
Minnesota	3	187		187
Missouri	4	72	50	122
New Hampshire	1		10	10
New Jersey	23	875	668	1543
New York	55	2574	2210	4784
Ohio	4	160	40	200
Pennsylvania	18	479	75	554
Rhode Island	2	45	180	225
South Carolina	1			
Texas	1	24		24
Virginia	1	28		28
Washington, D. C.	1		40	40
22 States	174	6076	4125	10201

elsewhere, as in convalescent homes. To find a basis for calculating the size of the home needed, Dr. Emerson proceeded as follows: The ten New York hospitals discharged 565 heart patients in one year. Assuming that they were still in need of care say for additional twenty to forty days per patient, a total stay of forty to eighty days is obtained. A one-hundred-bed hospital performing this service for 565

patients, the number of heart patients discharged in a year, could provide an average of 64.5 days per patient. Dr. Emerson goes on to say, "Of course the correct way of determining the extent of the need for beds for chronic heart patients would be by studying the disposition of these patients on discharge from general hospitals and inquiring into the adequacy of home or other care provided."

There are meanwhile about New York, 312 beds the year 'round available for heart patients in institutions, the total capacity of which is 910 beds; that is to say 34.2 per cent of the beds in eleven convalescent homes.

In the country at large, the convalescent home has likewise been developed. Whether the number of beds which they offer suffice for the local requirements I do not know. There are 174 homes in 22 states, with a capacity of 10,201, about 60 per cent (6,076) being available all year, the remainder (4,125) for the summer only. They provide for cases of maternity, orthopedic defects, tuberculosis, a few only exclusively for cases of heart diseases.

Meanwhile, with the decline in the death rate and in morbidity from tuberculosis, the expectation should be realized, that of the large numbers of beds provided for tuberculosis, a certain fraction would be relinquished for heart disease. G. J. Drolet[69] the statistician of the New York Tuberculosis Association, in compiling the resources of the country concludes that this is not the case, but that on the contrary, the country is in arrears, 25,864 beds. He bases his computation on the fact that there were 99,579 deaths from tuberculosis (1924) and only 73,715 beds. Presumably the deficit he cites, leaves out of account those cases which find care at home, those which are provided for in institutions for the insane (5,500) and those for prisoners in state institutions (1,300). It might be pointed out that the figures Drolet cites are based on the rate of one institutional bed for each patient; but he has pointed out the fact that this is an arbitrary arrangement especially in view of the fact that "by actual count only 20 per cent of the deaths from tuberculosis in the United States take place in institutions." Nevertheless it has been found that the beds can be kept continuously filled. In 1925, patients in tuberculosis hospitals were found to stay on an average, close to six months, namely, one hundred and seventy-four days.[69] But in general it appears that beds for heart

disease cannot be acquired from beds fallen vacant from use for tuberculosis, even provided that hygienically, this could be accomplished. There are states, however, in which beds for tuberculosis are in excess of the apparent needs, as for instance in Arizona, Colorado, Connecticut, Massachusetts, and New York. In other states there is, however, a deficit, as for instance in Maryland, California, Ohio, and Pennsylvania. What arrangements it is possible to make in the interest of the transfer that has been suggested it might be important to investigate.

I trust enough has been said to show that, from practically every point of view, information is inadequate, so far unavoidably inadequate. Geographical distribution, etiology, age relation, infectivity, heritability, therapeutics—wherever the subject is touched there is uncertainty. Because of the general chaos in which the whole matter of heart disease lies and because of the general lack of widespread information, there has been formed the association to which I have already referred. In New York, where the pioneering has taken place, alongside of the heart association, there arose cardiac clinics which also formed an association. There are now upwards of fifty of these clinics well distributed through the greater city. Next, the association and the clinics united and finally all were absorbed by the New York Tuberculosis and Health Association. Parallel with the New York City movement, a national association has been formed. The Heart Committee, as that part of the New York Tuberculosis Association is called which deals with heart disease, has as one of its committees a Committee on Research. This Committee has labored for several years with methods for collecting accurate and adequate data on the more general aspects of these diseases. It struggled for several years with devising forms[70] of reporting and believes that the method which was developed is adequate to its purpose. The forms which were produced are used in the Bellevue Heart Clinic, the largest one in New York, and have served as the basis of a number of the statements I have been able to make in this report. If you were to look through the publications dealing with certain aspects of this subject, you would I think be surprised to learn how much information has flowed from this single source. If it is possible to study cases in this manner at the Bellevue Clinic, it is, as we know already, possible elsewhere.

Even with slender resources, this investigation has gone forward, with a very small permanent staff.   With a grant recently acquired from the Commonwealth Fund, this study after its period of trial is to be expanded so as to include more clinics.and to be more broadly based. Great effort has been made to safeguard at the source the value of the data.   Agreement has been attained on nomenclature and classification, definition, and diagnosis.   Trained secretaries see to it that duplicate records from the various clinics are not brought together for collation until the record of each case has been edited and revised. The original records are the property of the clinics and are retained by them for whatever use the clinics desire.   Were the data entered for which there is provision on the record forms, each phase of the whole problem which I have been discussing could receive adequate exposition, instead of the fragmentary information I have been able to lay before you.   Accuracy of information can be attained only by this or by some other similar method.   But the pursuit of the aim will lay considerable obligation on the public-mindedness of those physicians who participate in this enterprise.

In the course of this exposition, I referred to the curves of Wyckoff and Lingg in considering which there developed a danger encountered in drawing the morbidity curve, first on account of dilution among the younger and second on account of paucity of cases among the older age groups.   These are difficulties which can be avoided; there should be sought methods of compensating the defect, especially in the older age group.   A utilization of the records in private practice can perhaps supply this need.   That this method is practicable has already been demonstrated in New York, and that this example be imitated is distinctly desirable.

It has been the purpose of this report to place emphasis on the meaning for the public health of certain aspects of the heart diseases and of their occurrence.   The most desirable plan for drawing attention to matters of this nature utilizes the statistical method; and this would also be the best method in a perfect world.   Under the circumstances the use of the method has revealed how imperfect a world this is for just this purpose.   But however imperfect the method, no other one is designed to present the facts.   To eschew the method because the results are defective and to fly to an extreme in which

the classification of cases is neglected but in which individual ones are described meticulously instead, begs rather than meets an important issue. Individual cases adequately and completely described remain without meaning from the point of view of probability until they are assembled in groups. But statements like this are elementary and would scarcely require mention were it not for the fact that, especially among the best observers, the fact escapes notice that data as data remain sterile; not until they are brought into action in a larger world of relevance do they take on significance. That accumulations of facts such as I have assembled leave much to be desired is not surprising; it is on the contrary encouraging to reflect that a sound beginning has been made and that ways are being devised for improving the methods of acquiring and of tabulating knowledge of this sort.

SUMMARY.

It is important to review certain results which issue from this inquiry.

1. Attention should perhaps first be drawn to the fact that unlike tuberculosis, heart disease concerns an organ and not a simple etiological moment. Diseases of the heart result from a number of different injuries. In the case of the heart, three main groups can be distinguished: first, that associated with rheumatic fever; second, syphilis; and third, arteriosclerosis or as I prefer to call it the senescent process.

2. There can no longer be doubt of the fact that rheumatic fever affects persons in the first three decades predominantly; that syphilitic heart disease is rare before the third decade and is often delayed two decades more; and that if due allowance is made for the age of occurrence and for the duration of these two disease types, the preponderant number of deaths occurs after age forty.

3. It is borne out by study of the curves which relate infectious and heart diseases, that the two have moved in opposite directions. The curve which represents the former has been falling, while that of the latter has risen. But the relation is not quite simple, for neither curve travels a smooth course. It is, however, possible to notice that at certain points, changes in the direction of both curves have taken place. It is furthermore noticeable that there occurred a delay in the

increased rise of the curve from heart disease for a number of years after that of the infectious diseases began to fall.   That this period is seventeen years may have a meaning but it is not an obvious one and no insistance is placed upon it.   That there should be a delay like this is easily comprehended; if individuals fail to die of infection, they will die in some other manner, and of affections which develop or are incident to life at later ages.

4. That the data which have been accumulated support this view seems clear, for the curves all show that the increased rate of death from the heart diseases involves persons over age forty.   Under this age, the rates, as these curves show have actually fallen.   Additional confirmation of this view resides in the fact that the rate increases with each decade.

5. The inference to be drawn from these statements has an important bearing on the fears which have arisen on account of the obvious increase in the death rates due to the heart diseases.   If as the data show, the increase involves the older age groups only, the result is altogether gratifying.   If men are, as the old adage has it, as old as their arteries, it becomes an important consideration to learn how long these may live.   If they live their full span of years, death from old age and death from circulatory defect may prove to be the same thing.   To die so would turn out to be the method of choice for the human organism; at all events, to realize it appears to be the direction which the course of events I have been describing seems to be taking.

6. Environment, climate, latitude all appear to influence the incidence and course of rheumatic heart disease.   But the data inspire no great confidence and suggest that the conditions under which they are collected be more precisely defined.

7. Experiences on the subject of treatment have been described. Apparently, from a remedial point of view, it is only in the rheumatic variety of heart disease that success can be anticipated from the use of methods designed to close the avenues of infection.   The suggestion that patients be treated, as are the tuberculous, with continuous prolonged rest, deserves consideration.   Too little is known now to justify the adoption of an opinion on this point.

CONCLUSION

It was my intention to show in this very incomplete summary that from the point of view of the public health, the problem of heart disease is not simple as was that of tuberculosis, but manifold. It includes disability from infectious diseases such as rheumatic fever and syphilis, and disability incident to the process of aging. The effort to relieve rheumatic fever by removing areas of infection still wants prosecuting before a judgment on its value can be given. To prevent the onset of the disease is still impossible. Theoretically the situation in syphilis is more fortunate; practically the methods of prevention and treatment offer enormous difficulty. Nor has a final conclusion been reached on the value of treatment. There also more knowledge is wanted. The disabilities dependent solely on age, the major aspect of the problem, must elude approach on the side of etiology; to provide relief from distress must be the direction in which to expend effort.

If the infectious aspect of heart disease is relatively speaking small, smaller than is often thought, that is a happy discovery. But that the rate of so-called chronic heart disease is high and is constantly mounting, that is a condition in which those may take satisfaction who believe increased length of life for more persons is one of the great blessings of men. The rise may be alarming but it is not malign.

So many matters are, as I have tried to indicate, not clear, that there must be general agreement that the idea which underlies the effort to accumulate information is sound and its prosecution desirable.

I should like to conclude these remarks with an expression of the pleasure I felt at receiving your invitation to make them as well as of the honor I know it to be, to be included among those who have had the opportunity to deliver De Lamar Lectures.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN THE BLOOD.

IX. The Distribution of Electrolytes between Transudates and Serum.

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This paper has for its purpose the presentation of data obtained from the analysis of blood serum and transudates from human subjects, and an examination of these data to determine whether the distribution of electrolytes in these systems is adequately defined by the laws first stated by Willard Gibbs, afterwards elaborated and tested by F. G. Donnan, and lately applied to protein solutions by Jacques Loeb. Van Slyke, Wu, and McLean showed in 1923 that the unequal distribution of bicarbonate and chloride ions between the serum and erythrocytes of horse blood, equilibrated at fixed CO_2 tensions, was in harmony with the ratios predicted by the Gibbs-Donnan law. Calculations made at that time from the data of Loeb, Atchley, and Palmer of the distribution of electrolytes between serum and transudates suggested that these were systems which were in approximate equilibrium and to which the Donnan law could be applied. Except in the case of the chloride ratios, however, discrepancies of considerable magnitude existed between the distribution found by analysis and that predicted by the formulæ developed by Van Slyke, Wu, and McLean. The data in this paper present further experimental evidence on the question of whether serum and transudates constitute a heterogeneous system in approximate thermodynamic equilibrium; and whether the membrane separating them is freely diffusible to inorganic anions and cations as is collodion, or whether it has properties of selective permeability as has the red cell membrane.

The system with which we are concerned may be schematically represented as in Fig. 1.

The symbols designate the following quantities:

$(B^+)_s$ = molal concentration of base in the serum.

$(B^+)_f$ =   "   "   "   "   "   " transudate.

$(A')_s$ =   "   "   " anions other than protein in the serum.

$(A')_f$ =   "   "   "   "   "   "   "   "   " transudate.

$(P)_s$ = protein concentration in the serum in gm. per liter.

$(P)_f$ =   "   "   "   " transudate in gm. per liter.
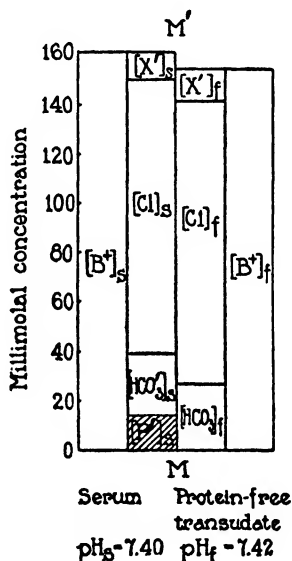
FIG. 1. Schematic representation of the distribution of electrolytes between serum and a protein-free transudate.

$(HCO_3')_s$ = molal concentration of bicarbonate in the serum.

$(HCO_3')_f$ =   "   "   "   "   "   " transudate.

$(Cl')_s$ =   "   "   " chloride in the serum.

$(Cl')_f$ =   "   "   "   "   "   " transudate.

$(X')_s$ =   "   "   " other anions in the serum.

$(X')_f$ =   "   "   "   "   "   "   " transudate.

The membrane MM' separating the serum and transudate is assumed to be freely permeable to all ions except the protein anions. If the system is in thermodynamic equilibrium then the distribution of

diffusible monovalent ions on opposite sides of the membrane is

(1)
$$r_{sf} = \frac{(A')_s}{(A')_f} = \frac{(B^+)_f}{(B^+)_s}.$$

But $(B^+)_s = (A')_s + (BP)_s$ where $(BP)_s$ designates the base bound by the protein and

$$(B^+)_f = (A')_f + (BP)_f. \quad \text{Furthermore} \quad (A')_f = \frac{(A')_s}{r_{sf}}.$$

Substituting these values in equation (1) for $(B^+)_s$, $(B^+)_f$, and $(A')_f$, and solving for r_{sf}, one obtains the expression

(2)
$$r_{sf} = \frac{(BP)_f + \sqrt{(BP)_f^2 + 4(A')_s\,[(A')_s + (BP)_s]}}{2((A')_s + (BP)_s)}.$$

If the protein concentration in the transudate is negligible, this simplifies to

(3)
$$r_{sf} = \sqrt{\frac{(A')_s}{(A')_s + (BP)_s}}.$$

The amount of base bound by protein, BP, is a function of the protein concentration and the pH. For horse serum it was found by Van Slyke, Wu, and McLean to be approximately expressed by the empirical equation:

(4)
$$(BP)_s = 0.068\,(P)_s\,(\mathrm{pH}_s - 4.80).$$

Since the titration curves of serum albumin and serum globulin differ greatly, and since the concentration of albumin and globulin are not the same in man as in the horse, the above expression cannot be used to calculate the base bound by the proteins of human serum. It was necessary, therefore, to determine the base bound by human serum proteins over the physiological pH range. This was done on dialyzed human serum in the manner described by Van Slyke, Wu, and McLean. The result of these experiments, which will be published in detail shortly, led to the following empirical relationship.

(5)
$$(BP)_s = 0.097\,(P)_s\,(\mathrm{pH}_s - 5.26).$$

Equation (5) will be used wherever it is desired to calculate the base bound by protein in human serum.   Equation (4) will be used in calculations based on horse serum analyses.

EXPERIMENTAL.

Our experimental results are of two types: (1) those obtained from analysis of the serum and transudates of pathological human subjects, and (2) those obtained from analysis of horse serum brought into equilibrium across a collodion membrane with a salt solution approximating a protein-free transudate in composition.

(1) The Electrolyte Distribution in Vivo.

Technique.

The transudates were collected under oil, then transferred to glass containers where they were kept over mercury until analyzed.   The blood from the vein of the unligated arm was collected under oil, centrifuged under paraffin, and the serum was transferred to a glass vessel where it was kept over mercury until analyzed.   *Carbon dioxide* content was determined in the manometric blood gas apparatus with an accuracy of 0.05 millimols (8); *chloride concentration* was determined by the wet ashing method (7); *the bases,* sodium, potassium, calcium, and magnesium were determined by the methods of Kramer and Tisdall; *phosphates* were determined by the method of Tisdall; *total base* was determined by a modification of the method described by Van Slyke, Wu, and McLean; *total protein* was determined by the Kjeldahl method; H_2O determinations were made by drying a known volume to constant weight at 110°C.; and pH was determined electrometrically. $BHCO_3$ was calculated from pH and total CO_2.

Results.

The results of the analyses of the serum and transudates obtained from three patients are given in Tables I, II, and III.   In the case of the subject of Table I analyses of edema fluid from the legs, and of ascitic fluid were obtained.   Since the ascitic fluid had about 40 per cent as much protein as the serum, whereas the edema fluid had only about 4 per cent as much protein, it was possible to make a comparison

of the effect of protein concentration on the electrolyte distribution in the same individual.   The distribution ratios predicted from equa-

TABLE I.

The Distribution of Electrolytes between Serum, Ascitic Fluid, and Edema Fluid.
Subject: De — cardiac decompensation.

	Serum	Ascitic fluid	Edema fluid
	gm./cc.	*gm./cc.*	*gm./cc.*
H₂O.................................	0.924	0.964	0.988
	gm./kg. H₂O	*gm./kg. H₂O*	*gm./kg. H₂O*
Total protein.........................	66.2	27.6	3.00
Albumin.............................	30.1	—	—
Globulin.............................	36.1	—	—
	mм/kg. H₂O	*mм/kg. H₂O*	*mм/kg. H₂O*
Total CO₂............................	30.8	31.8	31.8
BHCO₃..............................	29.3	30.2	30.4
Cl...................................	118.8	118.0	120.0
PO₄..................................	1.3	1.2	1.2
Na...................................	166.8	155.0	156.2
K....................................	6.0	—	4.1
Ca...................................	2.8	2.0	1.6
Mg...................................	0.7	0.5	—
pH (electrometric)..................	7.42	7.41	7.46
H⁺..................................	3.8×10^{-8}	3.9×10^{-8}	3.47×10^{-8}

Distribution of ions between:		Serum and ascitic fluid	Serum and edema fluid
		ratio	*ratio*
$(BHCO_3)_s : (BHCO_3)_f$.................	—	0.971	0.965
$(Cl)_s : (Cl)_f$...........................	—	1.007	0.991
$(Na)_f : (Na)_s$..........................	—	0.928	0.937
$(K)_f : (K)_s$............................	—	—	0.683
$\sqrt{Ca_f} : \sqrt{Ca_s}$......................	—	0.846	0.756
$(H)_f : (H)_s$............................	—	1.02	0.913
Calculated r_{sf}......................	—	0.975	0.958

tion (3) were r_{sf} = 0.975 for the system, serum : ascitic fluid, and r_{sf} = 0.958 for the system, serum : edema fluid.   The analytical ratios approaching these most closely were those for BHCO₃.

An error in the chloride analysis of serum led to an abnormally high chloride ratio. Ratios departing widely from the predicted were those for K, Ca, and Mg. As to the first named, there is known to be a relatively large error in potassium determinations of

TABLE II.

The Distribution of Electrolytes between Serum and Edema Fluid.

Subject: G—cardiac decompensation.

	Serum	Edema fluid
	gm./cc.	*gm./cc.*
H$_2$O	0.943	0.991
	gm./kg. H$_2$O	*gm./kg. H$_2$O*
Total protein	61.7	2.4
	mm/kg. H$_2$O	*mm/kg. H$_2$O*
Total CO$_2$	24.83	25.30
BHCO$_3$	23.54	24.12
Cl	110.3	114.3
PO$_4$	—	—
Na	140.5	133.9
K	6.4	4.9
Ca	2.3	1.5
Mg	0.6	—
pH (electrometric)	7.40	7.45
H$^+$	4×10^{-8}	3.55×10^{-8}

Distribution of ions between:		Serum and edema fluid
		ratio
(BHCO$_3$)$_s$:(BHCO$_3$)$_f$	—	0.976
(Cl)$_s$:(Cl)$_f$	—	0.966
(Na)$_f$:(Na)$_s$	—	0.954
(K)$_f$:(K)$_s$	—	0.77
$\sqrt{(Ca)}_f$:$\sqrt{(Ca)}_s$	—	0.81
(H)$_f$:(H)$_s$	—	0.89
Calculated r_{sf}	—	0.958

serum. Although the calcium analyses are probably correct, no allowance has been made for the combination of calcium with serum proteins. This normally amounts to about 1.0 mM. The amount of magnesium present is so low that sufficiently accurate analyses are impossible with present methods.

What has been said of Table I is equally true of Tables II and III when comparisons are made of electrolyte distribution between serum and edema fluid only. In these tables, the $BHCO_3$, Cl, Na, and H ratios approximate the predicted distribution ratios. It is of

TABLE III.

The Distribution of Electrolytes between Serum and Edema Fluid

Subject: D—nephritis.

	Serum	Edema fluid
	gm./cc.	*gm./cc.*
H_2O..	0.937	0.988
	gm./kg. H_2O	*gm./kg. H_2O*
Total protein....................................	4.48	—
	mM/kg. H_2O	*mM/kg. H_2O*
Total CO_2..	37.66	38.27
$BHCO_3$..	35.92	36.66
Cl..	99.5	101.9
PO_4..	1.22	1.00
Na..	147.5	136.8
K...	5.0	3.3
Ca..	2.1	1.4
Mg..	—	—
pH (electrometric)................................	7.43	7.47
H^+...	3.72×10^{-8}	3.39×10^{-8}

Distribution of ions between:		Serum and edema fluid
		ratio
$(BHCO_3)_s:(BHCO_3)_f$...........................	—	0.980
$(Cl)_s:(Cl)_f$....................................	—	0.976
$(Na)_f:(Na)_s$...................................	—	0.922
$(K)_f:(K)_s$.....................................	—	0.67
$\sqrt{(Ca)_f}:\sqrt{(Ca)_s}$	—	0.82
$(H)_f:(H)_s$.....................................	—	0.91
Calculated r_{sf}	—	0.969

interest to note that the subject of Table III was suffering from nephritis while the subjects of Tables I and II both were cardiacs. The electrolyte distribution picture of the former is, however, in no way different from the latter two.

The consistency of the observation that

$$\frac{(BHCO_3)_s}{(BHCO_3)_f} > \frac{(Cl)_s}{(Cl)_f} > \frac{(Na)_f}{(Na)_s} > \frac{(H^+)_f}{(H^+)_s}$$

suggests that the differences are real and not fortuitous. If the differences are not incidental to analytical technique, they indicate differences in the activity coefficients of the ions as pointed out in
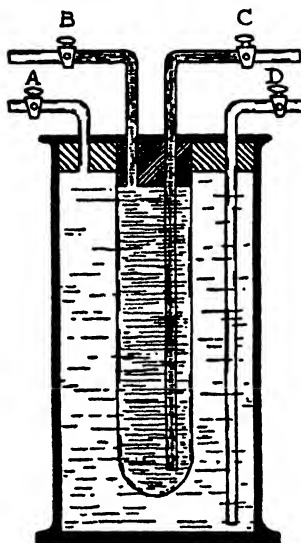
FIG. 2. The apparatus used for the dialysis of serum with salt solution. The serum was contained in the collodion bag suspended in the jar containing the salt solution. The serum was admitted through stop-cock C and withdrawn through stop-cock B. The salt solution was admitted through stop-cock D and withdrawn through stop-cock A. During dialysis all stop-cocks were closed and the whole kept in an air bath at 38°.

Paper VIII of the series on gas and electrolyte equilibrium in blood (9).

(2) The Electrolyte Distribution in Vitro.

Although it appeared from the above observations that the serum approaches equilibrium with such transudates as ascitic fluid and edema fluid from the extremities, and that the tissue separating the

TABLE IV.

The Distribution of Electrolytes between Horse Serum and a Salt Solution across a Collodion Membrane. Temperature 38°C.

Experiment No.	1		2		3	
	Serum	Dialysate	Serum	Dialysate	Serum	Dialysate
H₂O	*gm./cc.* 0.936	*gm./cc.* 0.990	*gm./cc.* 0.942	*gm./cc.* 0.993	*gm./cc.* 0.932	*gm./cc.* 0.990
Total protein	*gm./K₉H₂O* 72.0	*gm./K₉H₂O* 0	*gm./K₉H₂O* 66.2	*gm./K₉H₂O* 0	*gm./K₉H₂O* 70.8	*gm./K₉H₂O* 0
Total CO₃	*mм/K₉H₂O* 29.82	*mм/K₉H₂O* 30.27	*mм/K₉H₂O* 30.49	*mм/K₉H₂O* 30.82	*mм/K₉H₂O* 29.97	*mм/K₉H₂O* 30.62
BHCO₃	28.18	28.79	29.21	29.38	27.31	28.20
Cl	102.7	104.7	103.0	106.7	103.3	107.1
Na	145.3	126.7	144.0	133.6	143.1	130.8
K	6.8	5.4	6.6	5.7	7.4	6.0
Total base B	158.7	137.9	156.5	142.5	—	—
pH (electrometric)	7.34	7.385	7.45	7.50	7.15	7.21
H⁺	4.55×10^{-8}	4.12×10^{-8}	3.55×10^{-8}	3.16×10^{-8}	7.08×10^{-8}	6.17×10^{-8}

Distribution of ions between serum and dialysate

	ratio		*ratio*		*ratio*	
(BHCO₃)ₛ:(BHCO₃)f	0.979		0.994		0.969	
(Cl)ₛ:(Cl)f	0.982		0.966		0.966	
(Na)f:(Na)ₛ	0.871		0.934		0.914	
(K)f:(K)ₛ	0.794		0.86		0.81	
(H)f:(H)ₛ	0.906		0.891		0.872	
(B)f:(B)ₛ	0.868		0.910		—	
Calculated rₛf	0.958		0.960		0.961	

fluids behaves as though it were permeable to both anions and cations, it seemed desirable to study electrolyte distribution in a model system, having known properties of permeability.

An apparatus indicated in Fig. 2 was therefore devised which permitted serum and salt solutions to be separately prepared, saturated with CO_2 at any desired tension, brought into equilibrium at 38° across a collodion membrane permeable to electrolytes but not to proteins, again separately removed to containers over mercury, and analyzed.

Fresh horse serum was used throughout these experiments. The salt solution against which the serum was dialyzed had the initial composition: $NaCl = 100$ mM; $KCl = 6$ mM; $NaHCO_3 = 30$ mM; $CaCl_2 = 2.5$ mM; and was initially saturated at 38° with CO_2 at 40 mm. The dialysis was allowed to continue from 1 to 4 days without appreciable result upon the final outcome. The solutions were analyzed for H_2O, total base, total CO_2, chloride, sodium, potassium, protein, and pH by the methods already described.

The results of these experiments are given in Table IV. As in the case of the observations on human subjects, the $BHCO_3$, Cl, and Na ratios most nearly approximate those calculated from equation (2) and as before

$$\frac{(BHCO_3)_s}{(BHCO_3)_f} > \frac{(Cl)_s}{(Cl)} > \frac{(Na)_f}{(Na)_s} > \frac{(H^+)_f}{(H^+)_s}$$

These results confirm those of Loeb, Atchley, and Palmer in indicating that the distribution of ions between serum and transudates found in body cavities is governed by the same physicochemical laws which determine the distribution between serum and dialysates separated by a collodion membrane.

The results of the dialysis *in vitro* also indicate the probability that the differences in the distribution ratios of the different ions *in vivo* are not entirely attributable to the fact that the venous blood from the arm cannot exactly represent in its composition the capillary blood with which the transudates approach diffusion equilibrium. The differences in the ratios are partly attributable to factors inherent in the solutions, such as different activity coefficients of the respective ions.

SUMMARY.

1. Analyses have been made of the electrolytes and proteins of serum and transudates from human subjects.

2. The distribution ratios of HCO_3, Cl, Na, and H^+ deviated from unity as predicted by the Gibbs-Donnan law for similar heterogeneous systems.

3. Analyses of serum, and of artificial salt solutions approximating edema fluid in composition, after equilibration across collodion membranes showed distributions similar to those between serum and edema fluid *in vivo*.

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THE GASOMETRIC DETERMINATION OF SMALL AMOUNTS OF CARBON MONOXIDE IN BLOOD, AND ITS APPLICATION TO BLOOD VOLUME STUDIES.

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This paper presents a development of the blood carbon monoxide methods of Van Slyke and Salvesen (10) and of Harington and Van Slyke (4) with attention to certain details which reduce the error to 0.02 or 0.03 volumes per cent.

The present procedure was developed primarily to make possible the determination of blood volumes by the carbon monoxide method of Grehant and Quinquaud (3) without saturating, as has previously been necessary (1, 3, 6, 7), as much as one-third of the blood hemoglobin with CO in order to obtain accurate results. A technique requiring the saturation of only one-tenth to one-twentieth of the circulating hemoglobin makes the method more safe and convenient for use with human subjects, and with animals permits the introduction of the CO by intravenous injection of CO-saturated blood instead of by inhalation of the gas.

I. Determination of Small Amounts of Carbon Monoxide in Blood.

Reagents.

Acid Ferricyanide Solution.—This is the same solution used by Van Slyke and Neill for carbon monoxide in blood. It contains 3.0 gm. of Merck's saponin, 8.0 gm. of $K_3Fe(CN)_6$, and 40 cc. of 1 N lactic acid made up to 1 liter with water. It will keep for several weeks.

Pyrogallol.—10 gm. of pyrogallol are dissolved in a solution of 160 gm. of KOH in 130 cc. of water. For the CO determination described

below, this, because of its almost complete inability to dissolve either N_2 or CO gas, is preferable to hydrosulfite solution as oxygen absorbent.   The blood is removed from the chamber before the oxygen is absorbed, so that the solution does not gum the apparatus by precipitating the proteins.

Glycerol-Salt Solution.—1 volume of glycerol is mixed with 3 volumes of saturated NaCl solution.   This solution, because of its lower viscosity, is more satisfactory than the 1 to 1 mixture used by Harington and Van Slyke.   It is freed from air by extraction in the blood gas apparatus (see p. 535 of Van Slyke and Neill), and is stored under paraffin oil in a cylindrical separatory funnel, or, preferably, in the mercury bulb described on the same page.   If protected only by oil it must be used on the same half day that it is extracted, as the paraffin oil merely retards the access of air.   The solubility of air in this glycerol-salt solution is only a fraction of the solubility in water, but it is sufficient to necessitate the extraction before the solution is used for the present analysis.

Winkler's cuprous chloride solution for absorbing CO.   200 gm. of CuCl, 250 gm. of NH₄Cl, 750 cc. of water.   This solution also is freed of air not longer than 4 hours before use, and is kept under a layer of paraffin oil.   .

Technique of Analysis.—In the chamber of the blood gas apparatus described by Harington and Van Slyke (4) are placed 25 cc. of the acid ferricyanide solution and 6 or 7 drops of caprylic alcohol.   The air is removed by evacuating, shaking 3 minutes, ejecting the extracted air, and repeating the process once more.   5.5 cc. of the solution are forced up into the cup of the apparatus.   Then 5 cc. of blood are run under this layer of solution into the chamber of the apparatus from a pipette provided with a stop-cock and rubber tip, in the manner described by Van Slyke and Neill on p. 532 of their paper (9).   0.5 cc. of the ferricyanide from the cup is then run into the chamber to wash out the cock of the apparatus; the cock is sealed with a drop of mercury, and the 5 cc. of ferricyanide left in the cup are removed.

The mercury in the chamber is now lowered to the 50 cc. mark, and the apparatus is shaken for 8 minutes.   Because of the large volume of solution a longer time than the usual 2 or 3 minutes is needed to obtain

maximum extraction of the gases.   After extraction is finished the blood-ferricyanide mixture is drawn down into the bulb at the bottom of the chamber in two successive portions and ejected.

The chamber is then washed with two successive portions of about 5 cc. each of air-free glycerol-salt solution, as described by Harington and Van Slyke, to remove the blood-ferricyanide mixture.   The oxygen is then absorbed with pyrogallol as follows.   The glycerol-salt solution is removed from the chamber, and 5 cc. of pyrogallol are run into the cup of the apparatus.   The mercury in the chamber is so adjusted that a gas space of 3 or 4 cc. is present in the latter, and the stop-cock connecting manometer and leveling bulb is closed.   The pyrogallol is then admitted into the chamber in a slow trickle at the rate of about 0.5 cc. per minute.   The mercury in the manometer falls at first rapidly, then more slowly, as CO_2 and O_2 are absorbed. When, at the end of about 2 minutes, the rate of fall has become imperceptible, the cock leading to the leveling bulb is opened, and the rest of the 5 cc. of pyrogallol is admitted under slightly negative pressure. The cock at the top of the chamber is then sealed with mercury, and the final traces of oxygen are absorbed by raising and lowering the leveling bulb so that the pyrogallol rises and falls in the upper fourth of the chamber.   After it has been raised and lowered about 5 times the surface of the pyrogallol is brought to the 0.5 mark (or to the 2.0 cc. mark in case there is so much CO that it gives over 400 mm. pressure at 0.5 cc. volume), and the manometer is read.   The process and the reading are repeated until the latter becomes constant.   Complete absorption of the oxygen requires about 5 minutes in all.   The last trace must be removed with certainty, because if any oxygen is left it will be absorbed by cuprous chloride in the next stage of the analysis, and will be calculated as carbon monoxide.

After absorption of the oxygen, the pyrogallol is ejected through the trap at the bottom of the chamber, and the chamber is washed with two successive 5 cc. portions of glycerol-salt solution to remove the alkali, which would otherwise precipitate the cuprous chloride solution added later.

A third portion, this time exactly measured, of 5 cc. of glycerol-salt solution is then run in, and the manometer is read with the gas volume in the chamber at the original 0.5 or 2.0 cc.   The reading is

checked by lowering the solution in the chamber a little, and then bringing the meniscus back to the 0.5 cc. mark. Because of the time required for drainage of the glycerol-salt solution down the walls at the top of the chamber above the 0.5 or 2.0 cc. mark, the actual free volume is usually a little under 0.5 or 2.0 cc. at the first reading. The second manometer reading will then be a little lower, but the third or fourth will be constant. The final reading is noted as p_1. After the final reading the glycerol-salt solution is ejected through the trap at the bottom of the chamber. The p_1 reading over the glycerol-salt solution is used in preference to that over the pyrogallol, because the meniscus of the clear glycerol-salt solution can be located on the 0.5 or 2.0 cc. mark more accurately than the meniscus of the black pyrogallol solution.

To absorb the carbon monoxide, 6 cc. of the air-free Winkler's cuprous chloride solution are placed in the cup of the apparatus, and 5 cc. are run into the chamber at *slight* negative pressure, the mercury surface in the leveling bulb being only a few cm. below that in the chamber, and the cock open between the leveling bulb and the chamber (note remarks by Harington and Van Slyke on p. 582 of their paper). The cuprous chloride is admitted slowly enough so that 2 minutes are taken. In this time absorption of carbon monoxide is complete. The surface of the solution is now lowered carefully to the original 0.5 or 2.0 cc. mark and the pressure p_2 in the manometer is read. The reading may be checked once by lowering the solution in the chamber a few mm. and bringing it back to the mark. If several check readings are attempted, however, a gradual rise in the observed pressures will be noted, because the absorbed CO begins to return to the gas phase, on account of the loose, reversible character of the combination between CuCl and CO. The lowest reading of such a series is the correct one.

The time required for the entire analysis is about 30 minutes.

Calculation of CO Content of Blood.

The partial pressure of carbon monoxide, P_{CO}, in mm. of mercury is $P_{CO} = p_1 - p_2 + 2.0$.

The 2.0 mm. correction is due chiefly to the fact that the glycerol-salt solution has a vapor tension about 2 mm. lower than the cuprous chloride solution.

The CO content of the blood is calculated by multiplying the observed P_{CO} pressure by the proper factor from Table I, which is calculated by Van Slyke and Neill's Equation 6 with the factors in their Table I. In the analysis above described $a = 0.5$, or 2.0, $S = 25.0$, $i = 1.00$, and *cc. sample* = 5.0.

TABLE I.

Factors by Which P_{CO} Is Multiplied to Calculate Volume Per Cent of CO When 5 Cc. of Blood Are Analyzed.

Temperature.	Factor when P_{CO} is measured with gas at 0.5 cc. volume.	Factor when P_{CO} is measured with gas at 2.0 cc. volume.
°C.		
15	0.01277	0.0511
16	73	09
17	67	07
18	62	05
19	57	03
20	52	01
21	48	0.0499
22	43	97
23	38	95
24	34	94
25	29	92
26	24	90
27	19	88
28	15	86
29	11	84
30	06	82
31	02	81
32	0.01198	79
33	93	77
34	88	75

In the analysis as above described for each cc. of blood, 4 cc. of the acid ferricyanide solution are used instead of 2.5 cc., as prescribed by Van Slyke and Neill and by Harington and Van Slyke. We have found that in some samples of dog blood the larger amount is necessary.

II. The Carbon Monoxide Blood Volume Method with Intravenous Injection of the CO.[1]

Procedure.—For injection, sufficient blood to make somewhat over 4 cc. per kilo of body weight of the animal to be injected was defibrinated, strained through gauze, and saturated with carbon monoxide gas (made by heating anhydrous formic acid and concentrated sulfuric acid (1, 3)). A cylindrical separatory funnel, of 10 or 20 times the volume of the blood portion, made a convenient saturater. The blood was placed in the funnel, which was then evacuated from the cock as completely as possible by a water pump, and was filled with carbon monoxide at atmospheric pressure. The saturation was accomplished by rotating the funnel for a half hour in the manner shown by Stadie (8). With dogs one may draw the blood sample either from the animal whose blood volume is to be determined, or from another donor.

Part of the saturated blood was reserved for CO determinations on 2 cc. portions by the technique of Van Slyke and Neill. Since the O_2 and N_2 had been displaced from the blood by CO, only the gases CO_2 and CO remained. Consequently, in this analysis of the CO-saturated blood, the gases were extracted, the CO_2 was absorbed with 1 N sodium hydroxide, and the entire residual gas was measured as CO, the p_2 reading being taken after ejection of the CO gas. The absence of oxygen and nitrogen made the use of specific absorbents for O_2 and CO unnecessary.

Of the CO-saturated blood a measured volume approximating 4 cc. per kilo of body weight, and equal to about one-twentieth of the animal's blood volume, was injected intravenously in the course of 1 to 1.5 minutes. At intervals of a few minutes thereafter samples of about 20 cc. were drawn from another vein, without exposure to air, as described by Austin *et al.* (2).[2] 5 cc. portions were analyzed for CO content by the special technique described in this paper.

[1] In the procedure described the CO is injected in the form of blood saturated with the gas. Preliminary experiments have shown that intravenous injection of the gas itself is possible, provided the rate is sufficiently slow and uniform to permit absorption of the gas by the blood in the vein.

[2] According to Lee and Whipple, however, this precaution is unnecessary. The affinity of CO for blood is so great that significant loss does not occur even if ordinary contact with air in a tube is permitted.

In order to ascertain also the hemoglobin content of the blood, a portion of 7 cc. of the drawn blood was saturated with CO gas, as described above, and the CO content was determined as in the analysis of the injected blood. To calculate the CO bound by hemoglobin, the physically dissolved CO was estimated and subtracted from the total observed CO content. The physically dissolved CO was estimated on the assumption that the solubility of CO in blood is proportional to the water content of the latter, and is therefore on the average 82 per cent of the solubility in pure water. This assumption is probably not exact, but the degree of error introduced appears to be slight, as hemoglobin contents estimated from CO capacity, with this solubility correction, are the same as those estimated from O_2 capacity after saturation with air. The physically dissolved CO, in volumes per cent of the blood, is thus estimated to be at 15°, 2.16; at 20°, 1.95; at 25°, 1.82; at 30°, 1.71.

The value of the factor, $f = \dfrac{\text{cc. cell volume per 100 cc. blood}}{\text{cc. CO capacity per 100 cc. blood}}$, which is used in calculating the volume of blood cells in the body (see Equation 3 below) was determined as follows. A 10 cc. sample of blood was drawn into a tube containing 2 cc. of 1.6 per cent neutral potassium oxalate (isotonic for blood, as used by Lee and Whipple (5)). 7 cc. of the oxalated blood were used for determination of the CO or O_2 capacity, while the cell volume in other samples was determined by hematocrit. (Instead of blood treated with isotonic oxalate, defibrinated blood may be used to determine f. In this case the defibrination should be performed without loss of CO_2 in order to prevent the shrinking of several per cent in the cell volume which occurs if by reason of such loss the serum pH rises, as it may, to 8 (Van Slyke, Wu, and McLean (11)).

Calculation and Interpretation of Blood Volume Results.

The interpretation of the results of the carbon monoxide method has been made the subject of a thorough experimental and critical study by Whipple and his collaborators (1, 7). No one should apply this blood volume method without consultation of their papers, the conclusions of which will here be but briefly indicated in connection with the calculations.

CO-Binding Capacity of Total Hemoglobin.—Assuming that during the interval between injection of CO and withdrawal of the venous blood sample for analysis, (1) distribution of the injected carbon monoxide among the cells of the circulating blood becomes uniform, and (2) none of the injected CO leaves the circulation, either through the lungs or by diffusion into the tissues, one could calculate the CO-binding capacity of the circulating hemoglobin as follows:

(1) Cc. CO-binding capacity of total blood hemoglobin = (cc. CO injected) $\times$

$$\frac{\text{(volume per cent CO capacity of circulating blood)}}{\text{(volume per cent CO content of circulating blood)}}.$$

Distribution of the injected carbon monoxide does, in fact, seem to be completed in 3 or 4 minutes.

Loss from the lungs within this period appears, both from Whipple's results (1) and ours, to be negligible in this time.

Diffusion of CO from blood to tissues, however, is a factor concerning the rapidity and extent of which we have no conclusive data. As Whipple has shown (1, 12) the muscles contain considerable amounts of pigment which when extracted with dilute ammonia and saturated with CO behaves colorimetrically like hemoglobin. Whether in the animal carbon monoxide actually passes from the blood cells to combine with this pigment in the muscles, how rapidly, and in what proportion, are questions that await experimental solution.

In favor of the probability that during a period of 10 minutes but a small proportion of CO passes from blood to tissues, are the observations of Smith, Arnold, and Whipple (7) that the CO method gives *lower* total blood volumes than the dye methods. In their experiments an atmosphere containing CO was respired for 6 minutes, and the blood sample for CO determination was drawn 4 minutes later. The effect of passage of CO to the tissues would be to *increase* the dilution of CO in the blood, and the CO-binding capacity, blood cell volume, and blood volume, of the body estimated from the blood CO concentration.

Volume of Total Blood Cells in Body.—From the total CO capacity of the body the total volume of blood cells in the body is calculated as

(2) Cc. total blood cells in body = (cc. total CO-binding capacity) $\times f$.

The total CO capacity is estimated by Equation 1. The factor f is the ratio $\dfrac{\text{(cc. cell volume per 100 cc. blood)}}{\text{(cc. CO capacity per 100 cc. blood)}}$, which can be accurately determined as described above. The uncertainties of the red cell volume estimation are those of total CO capacity factor, discussed in connection with Equation 1.

Total Blood Volume.—The formula for estimating total blood volume is

$$(3) \quad \text{Cc. blood volume} = \frac{100 \times (\text{cc. CO injected})}{(\text{volume per cent CO in circulating blood})}.$$

This equation is based upon the assumptions of uniform distribution and negligible loss of CO, discussed in connection with Equation 1, and in addition a third assumption; *viz.*, that the volume per cent of cells is constant in all parts of the circulating blood. Smith, Arnold, and Whipple (7) give reasons for believing that this third assumption is not even approximately exact, that the peripheral blood samples drawn for CO analyses contain a larger proportion of hemoglobin than the average blood of the body, and that in consequence the denominator of the fraction $\dfrac{\text{CO injected}}{\text{CO content of blood}}$ is larger in the sample than in the average blood of the body. Presumably as a result of this lack of uniformity in the circulating blood, they obtained in dogs by the CO method total blood volumes about 20 per cent lower than they considered correct, when the results were compared with those of dye injection methods. They considered that the most accurate total blood volume was to be obtained by adding the cell volume yielded by the CO method (Equation 2 above) to the plasma volume obtained by the dye injection method.

Results.

The results of two experiments are given in Tables II and III. They indicate that the experimental variations can be limited to 5 per cent, and perhaps 3 per cent, by the method outlined.

Arnold, Carrier, Smith, and Whipple (1) have found that, during the first 4 minutes after a dog has been partially saturated with carbon monoxide through the lungs, and has begun again to breathe

normal air, the CO content of the blood does not diminish measurably, although an appreciable diminution does occur during the second 4 minutes. Our data obtained after intravenous injection of CO are similar. Besides the loss of CO from blood to muscle pigment by diffusion, one would expect excretion of the gas by the lungs to begin at maximum speed as soon as the animal respires air free from the gas, and we are at a loss to explain the apparent lag in excretion

TABLE II.

Dog, weight 12.35 kilos. Injected 50 cc. of another dog's blood with 20.45 volume per cent CO = 10.22 cc. of CO.

Time from injection to drawing blood sample.	P_{CO} from 5 cc. of blood at 0.5 cc. volume.	Temperature of gas at P_{CO} measurement.	CO concentration in blood.	CO content of body.	Blood volume calculated by Equation 3.		CO-binding capacity of blood.	Total CO-binding capacity of body calculated by Equation 1.	Estimated fall in CO capacity of body due to drawing of blood samples.
					Total.	Per kilo.			
min.	*mm.*	*°C.*	*vol. per cent*	*cc.*	*cc.*	*cc.*	*vol. per cent*	*cc.*	*cc.*
3	78.8	24	0.97	10.22	1054	85	13.8	145	
	81.0	23	1.00	10.22	1022	83	13.8	141	
6	79.5	23	0.98	10.02*	1028	83	13.6	139	2.0
	81.0	22.5	1.00	10.02	1007	82	13.6	136	
10	75.0	22	0.93	9.82*	1055	85	13.2	139	4.0
	74.0	23	0.92	9.82	1066	86	13.2	140	

* Corrected by subtracting from injected CO the amount withdrawn in preceding blood samples of 20 cc. each. This corrected value is used, in place of the total injected CO, in calculating by Equation 1 the figures in the next to the last column.

during the first 4 minutes. Nevertheless Whipple's blood analyses and ours indicate that the lag occurs.

The decreases that occur in the CO content in the second 5 minutes of our experiments are due in part to the removal of blood samples each amounting to 2 or more per cent of the animal's total blood. These removals decrease the CO-binding capacity of the body by the amounts indicated in the last columns of Tables II and III, and should

decrease the capacity estimated from the blood CO content by the same amounts if there were no losses of CO from the circulating blood. In the experiment of Table II it seems doubtful that appreciable loss

TABLE III.

Dog, weight 14.25 kilos. Drew and defibrinated 110 cc. of this animal's own blood. Saturated a portion with CO. Reinjected 50 cc. of blood of 19.06 volume per cent CO = 9.53 cc. of CO.

Time from injection to drawing blood sample.	P_{CO} from 5 cc. of blood at 0.5 cc. volume.	Temperature of gas at P_{CO} measurement.	CO concentration in blood.	CO content of body.	Blood volume calculated by Equation 3.		CO-binding capacity of blood.	Total CO-binding capacity of body calculated by Equation 1.	Total red cell volume in body calculated by Equation 2.*	Estimated fall in CO capacity of body due to drawing of blood samples.
					Total.	Per kilo.				
min.	mm.	°C.	vol. per cent	cc.	cc.	cc.	vol. per cent	cc.	cc.	cc.
3	102.0	23.0	1.26	9.53	755	52.5	14.7	111	247	0
	102.1	23.0	1.26		755	52.5		111	247	
5	102.8	22.5	1.27	9.28†	731	52.3	14.8	108	240	3.0
	100.3	27.5	1.24		749	53.6		111	247	
8	91.4	22.5	1.13	9.03†	798	56.0	14.0	112	249	5.5
10	87.3	22.5	1.08	8.80†	813	57.0	13.8	112	249	7.8

* f of Equation 2 was found to be 2.22. The combining capacity of the defibrinated blood was 19.06 − 1.84 = 17.22 volume per cent. The cell volume by three hematocrits was found to be 38.1, 38.0, 38.3, average 38.2 per cent of the blood volume. Therefore $f = \dfrac{38.2}{17.22} = 2.22$.

† Corrected by subtracting from injected CO the amount withdrawn in preceding blood samples of 20 cc. each. This corrected value is used, in place of the total injected CO, in calculating by Equation 1 the figures in the next to the last column.

occurred in the 10 minutes of the experiment. In the experiment of Table III the CO capacity of the body calculated from the analyses remained constant while enough blood was drawn to reduce it by 7 per cent. Apparently therefore a loss of about 7 per cent of the circu-

lating CO occurred in the 10 minutes of the experiment, chiefly in the second 5.

SUMMARY.

A technique is described for quantitative gasometric determination of small amounts of carbon monoxide in blood.

It appears that 4 minutes after injecting carbon monoxide blood as described in this paper one can estimate the volume of the circulating red cells from the blood CO content with less than 5 per cent error due to analytical technique and CO distribution within the blood. We have not ascertained the magnitude of the possible additional error due to diffusion of CO from blood to tissue hemoglobin.

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THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

III. FURTHER STUDIES ON THE NATURE OF THE PURPURA-PRODUCING PRINCIPLE.

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INTRODUCTION.

It will be recalled from a previous (1) communication that pneumococcus extract was found to produce hemorrhagic purpura in laboratory animals.[1] The purpura-producing principle resists oxidation and heating to 100°C. for 10 minutes; it is filter-passing; it is present in the fraction of pneumococcus extract obtained by full saturation with $(NH_4)_2SO_4$ after the acetic acid-precipitable substances have been removed. The principle is not associated with type specificity or virulence of the organism and is distinct from the endohemotoxin always present in reduced extracts of Pneumococcus (2). The opinion was expressed at the time that the purpura material was probably a degradation product of pneumococci, since it is present only when cell disintegration accompanied by autolysis has taken place.

Subsequent study (3), established the fact that pneumococcus extract is thrombolytic both *in vivo* and *in vitro*, and that the development of purpura in mice following the injection of pneumococcus extract is associated with an excessive diminution in the number of blood platelets, the greatest decrease usually occurring within 24 hours. In addition the red blood cells also are greatly reduced numerically,

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[1] Since our preceding paper, we have seen a reference of Carnot (Carnot, P., *Compt. rend. Soc. biol.*, 1899, li, 927) in which he describes reactions in rabbits to a "pneumococcus toxin." His descriptions indicate the development of purpura in these animals.

but the rate of their disappearance is slower than that of the thrombocytes. Although heat inactivates both the thrombolysin and hemolysin, it has no effect on the purpura-producing property of pneumococcus extract. Adsorption of extract with blood platelets reduces the activity of the thrombolysin, but does not influence the purpura-producing activity in animals.

In the present communication, further studies are reported upon the nature of the purpura-producing principle. Observations are recorded on (1) the immunological reactions, (2) the biological significance and (3) the further chemical fractionation of this substance in pneumococcus extracts.

I. Immunological Reactions of the Purpura-Producing Principle.

(a) *Antigenicity of the Purpura Material.*—The purpura-producing substance, fractionated as will be described later, was used as immunizing antigen in rabbits. It was noted that during immunization, purpura lesions which accompanied the earlier injections, were not induced by the succeeding inoculations of the material. After several weeks of immunization, the sera were tested for neutralization of purpura in white mice and for the presence of pneumococcus antibodies. Not only did these sera not possess the power of neutralizing the purpura substance, as is brought out below, but they contained no antibodies for the intact cell or its derivatives. Thus, precipitins were not demonstrated for the purified purpura principle; for the soluble specific substance of the homologous type of Pneumococcus, nor for either extract or protein of the cell. Similarly, it was not possible to show agglutinins for either the "S," encapsulated cell, or the "R," capsule-free cell; nor were the sera able to confer protection upon mice against infection with homologous or heterologous types of Pneumococcus. In this respect, the purpura principle differs markedly from the hemotoxin, since the latter is antigenic (2, 4).

(b) *Active Resistance to the Purpura Principle.*—The rabbits employed in the foregoing experiment indicated that notwithstanding the fact that the purpura substance is non-antigenic, an increased resistance may be acquired against purpura. In order to study more thoroughly whether resistance can be exhalted actively, mice were treated with heat-killed suspensions of Pneumococcus, or with

the protein (5), autolysate (1) or extract (6) of the bacterial cells. All the substances are known to stimulate the formation of antibodies (7) and the last two contain the purpura principle. Accordingly, mice received five weekly injections of the particular antigen either subcutaneously or intraperitoneally. 7 to 10 days after the final injection, the mice were tested for increased resistance to purpura.

TABLE I.

Increased Resistance to Purpura Acquired by White Mice Following Repeated Injections of Pneumococcus Extract.

Date of injection	Amount of extract injected	Number of mice injected	Number of mice developing purpura
	cc.		
Apr. 26	0.1	10	10
May 3	0.1	10	10
May 10	0.2	10	8
May 17	0.2	10	2
May 24	0.4	10	0
June 1	0.5	10	0

TABLE II.

The Protective Action of Immune Sera against Purpura in White Mice.

Sera	Number of mice injected	Mice developing purpura	
		Number	Per cent
Controls (no serum)........................	10	7	70
Normal serum.............................	20	16	80
Antipurpura..............................	10	10	100
Antipneumococcus.........................	5	5	100
Anti-R...................................	15	7	46
Anti-extract..............................	25	13	52
Anti-autolysate...........................	25	21	84
Antiprotein..............................	20	14	70

It was found that repeated injections of pneumococcus vaccine or protein do not increase the tolerance of mice to purpura. Mice so treated developed purpura following the injection of the same amount of extract as was required for the production of purpura in normal animals. Immunization to pneumococcus extract or autolysate, on the other hand, definitely renders mice more resistant to pur-

pura. This fact has been demonstrated satisfactorily a number of times, and a protocol typical of the results (Table I) shows that ten mice were made refractory to purpura by repeated injections of increasing amounts of pneumococcus extracts. It is also of note that the purpura substance no longer causes a diminution in the number of blood platelets or red blood cells in mice whose tolerance to purpura has been raised.

(c) *Passive Protection against the Purpura Principle.*—Whether white mice can be passively protected against purpura, was studied by the use of a number of immune rabbit sera prepared by immunization with the purpura principle itself, with "S" and "R" strains of Pneumococcus and with cell extracts, autolysates and the nucleoprotein fraction of the organism. In determining the neutralizing action of the sera against purpura, mixtures of 0.5 cc. of serum and 0.1−0.2 cc. of extract were injected intraperitoneally either immediately on mixing or after incubation together for 1 hour at 37°C. The sera prepared by immunization with the purpura material did not prevent the occurrence of purpura in mice. In no instance, moreover, did any of the sera provide definite passive protection against purpura. The data in Table II show the variation in susceptibility of individual mice and consequently the difficulty of evaluating experiments of this kind. While in the present instance 70 per cent of the normal mice developed purpura, other observations have shown variations from 100 per cent (*cf.* Table I) to 30 per cent of normal mice reactive to a fixed dosage. However, it is sufficiently clear that the sera included in the study did not confer upon normal mice protection against purpura. The lower percentage of animals developing purpura when anti-extract and anti-R sera were utilized, is suggestive of a partial protection, but experience with the purpura substance makes more plausible the interpretation of variation in susceptibility.

The evidence obtained, therefore, is that the purified purpura principle is non-antigenic, in the sense that it produces no demonstrable antibodies. White mice injected repeatedly with substances containing the purpura material acquire an increased tolerance to purpura. On the other hand, the sera of rabbits immunized with the purpura principle, the intact cell or its derivatives confer upon white mice no passive protection against purpura.

II. *Biological Nature of the Purpura-Producing Principle.*

It was shown in a preceding paper (1) that the purpura substance was found only in those cell derivatives which represented degradation products of pneumococci. The evidence thus far indicates that the substance is not contained as such in the cell, but rather that the purpura principle is a product accompanying cell autolysis. The present experiments substantiate this point of view and confirm the autolytic origin of the purpura material.

Attempts to obtain the purpura substance by dissolution of the cell with bile were repeated, but again the bile solutes were found incapable of producing purpura. Pneumococci were dissolved also by means of sodium oleate, but the resulting solutions did not contain the purpura principle. Curiously enough, incubation of bile solutes to allow digestion by the autolytic enzymes present in the cell did not yield the purpura substance.

Extracts prepared by rapidly freezing and thawing heavy suspensions of live pneumococci are actively purpura-producing, and there is reason to believe that this activity is dependent upon the action of the enzymes of the cell. Since the enzymes of Pneumococcus are inactivated (8, 9) by heating, whereas the purpura principle is resistant to heat (1), it was possible to determine the rôle of the enzymes in the derivation of the purpura substance. Extracts were prepared by freezing and thawing pneumococci previously exposed to 100°C. for 5 minutes. The extracts prepared from heat-killed cells, however, were found to be incapable of producing purpura in white mice. This is further evidence that the purpura principle is not preexistent in the cell.

Avery and Cullen (8, 9) have shown that cell-free filtrates of Pneumococcus contain, among other enzymes, a potent bacteriolytic enzyme which is operative on a substrate of heat-killed pneumococci. It became possible therefore to analyze the relationships of autolytic digestion to the formation of the purpura principle. A solution of active pneumococcus enzymes prepared according to the method of Avery and Cullen was allowed to act on cells killed by heating for 5 minutes at 100°C. After digestion at 37°C., there was distinct lysis of the organisms and cell-free filtrates were obtained after 24, 48,

72 and 96 hours.   The filtrates, when injected into white mice, were found to be actively purpura-producing.   The enzyme-containing extract, when diluted to the same extent as the filtrates obtained from the digestion experiment, did not produce purpura.

A pneumococcus enzyme solution was inactivated by boiling.   It was then allowed to act on heat-killed pneumococci as in the above experiment.   No lysis was observed, however, and the filtrates obtained from these tests did not produce purpura.   Similar tests conducted with active enzymes in the presence of bile showed that the purpura substance was not demonstrable under these conditions.

The evidence indicates definitely, therefore, that the . purpura principle is not a preformed constituent of the cell, but that it is a product of autolysis.

Avery and Cullen (9) have shown that the enzymes of Pneumococcus cause lysis not only of Pneumococcus itself, but also of *Streptococcus viridans*.   It was desirable, therefore, to determine whether purpura-producing properties could be obtained from streptococci by digestion with pneumococcus enzymes.   Heavy suspensions of *Streptococcus viridans*, killed by heating at 100°C. for 10 minutes, were subjected to the action of pneumococcus enzymes as described in the preceding experiment.   Filtrates obtained from the resulting bacteriolysis of streptococcus by pneumococcus enzymes were not capable of producing purpura in white mice.

Purpura is rarely seen in white mice during the course of experimental infection with Pneumococcus.   This is possibly due to the fact that the virulent organisms are tremendously invasive and cause death rapidly.   Since degraded and avirulent "R" cells yield as potent purpura-producing extracts as their virulent antecedents (1), a study of the disintegration of pneumococci provided a means for determining the occurrence of purpura following the injection of live pneumococci *in vivo*.   Small and large amounts of concentrated suspensions of live "R" cells were injected both subcutaneously and intraperitoneally in mice.   The majority of the mice survived but in no instance was purpura observed.

In summary, then, it can be said that the purpura-producing principle of Pneumococcus does not exist preformed in the cell.   It definitely accompanies autolysis *in vitro* and, under the conditions

stated, purpura was not observed in animals injected with large amounts of live pneumococci.

III. Chemical Nature of the Purpura-Producing Principle.

Previous studies indicated (1) that the purpura substance was precipitated by full saturation with $(NH_4)_2SO_4$ after the nucleoproteins had been previously removed from pneumococcus extract. It was not clear, however, whether the purpura material was albumin or not. In the present study, further fractionation has been accomplished and the purpura principle has been separated from the original precipitate obtained with $(NH_4)_2SO_4$.

In the chemical purification, aqueous extracts of Pneumococcus were employed. Normal acetic acid was added to the extract until no further precipitation occurred. The acid precipitate was removed by centrifugation and the supernatant was withdrawn. The supernatant was heated to maximum coagulation, and the coagulum was whirled down. The materials removed by acid and by heat, as well as the supernatant after removal of both proteins, were studied.

As was previously shown (1) the acetic acid precipitate or nucleoprotein was not purpura-producing. The heat-coagulable proteins were irregular in their action and at best only faintly reactive. Since they were not washed, it seems likely that they were not entirely free from the supernatant. The supernatant remaining after both the acid-precipitable and heat-coagulable proteins had been removed, usually produced marked purpura in mice. The indications are therefore that the purpura principle is not present either in the acid-precipitable or heat-coagulable proteins.

The active purpura-producing supernatant as such gave no qualitative color tests for protein. When it was concentrated to one-tenth volume however, several tests were obtained which aid in identification of the principle. With the biuret test, a pink to purple color was observed. The xanthoproteic test was negative, and Millon's reagent gave a yellow to brown precipitate. A precipitate was obtained with full saturation of $(NH_4)_2SO_4$; the precipitate first appeared at about 60–70 per cent saturation and it increased in intensity up to full saturation. Both picric acid and trichloracetic acid gave precipitates, but in both instances the precipitates vanished

on heating and returned on cooling.   Since the concentrated solution, moreover, gave no further coagulation on boiling, it appears that the purpura-producing principle of Pneumococcus is identified with a primary proteose.   It cannot be said whether it is actually a proteose or some closely associated substance.   The purpura material is present in such small amounts and some is lost during fractionation, so that it becomes a difficult procedure to effect a further separation.

DISCUSSION.

The experiments recorded in this communication disclose that the purpura-producing principle of Pneumococcus does not stimulate the formation of demonstrable antibodies.   The serum of animals immunized with either the purpura principle itself, with pneumococcus cells or with extract, autolysate or nucleoprotein of the bacteria does not afford white mice passive protection against purpura.   In contrast to this fact, however, white mice and rabbits acquire an increased tolerance to purpura following successive injections of materials containing the purpura substance.   It seems likely, therefore, that the "active immunity" in this instance is really an increased resistance to the toxic agent.   This is supported by two facts: (1) the purpura-producing principle is not antigenic, and (2) passive protection against purpura is not transferred to normal mice by the serum of resistant animals.

The experiments on the biological nature of the purpura principle furnish direct evidence that it accompanies autolysis and that it is most probably a degradation product.   It was not obtained as a preformed constituent of the cell, since, in the absence of enzyme action, cytolysis of live or dead cells did not yield the purpura substance.   Dissolution of the live cell by bile or sodium oleate, neither of which inhibits the activity of the purpura principle when once formed, did not furnish the purpura material.   Moreover, the presence of bile has been observed to inhibit definitely the formation of the purpura substance.   In fact Jobling and Strouse have shown that a number of unsaturated fatty acids, including sodium oleate, actually do inhibit the action of proteases.   Since bile does contain unsaturated fatty acid, there is some support for the belief that bile inhibits the digestive processes which give rise to the purpura prin-

ciple.   Freezing and thawing heat-killed organisms in the absence of active enzymes did not liberate the purpura principle.   On the other hand, the digestion of dead cells by active enzymes of Pneumococcus gave filtrates which were actively purpura-producing.   In other words, the purpura substance appears to be an autolytic derivative of Pneumococcus.

Further fractionation of the purpura material indicates that it is closely related to a primary proteose.   It has been definitely separated from both the acid-precipitable and heat-coagulable proteins of pneumococcus extract, but a more exact identification cannot be stated at the present time.   In general proteoses have been found to be non-antigenic (10) although the observations of some investigators notably those of Fink (11) indicate that proteoses may be antigenic.   That proteoses, moreover, may be toxic has been demonstrated by a number of investigators (12).

CONCLUSIONS.

1. The purpura-producing principle of Pneumococcus is non-antigenic in the sense that it does not stimulate the formation of antibodies.

2. White mice acquire an increased resistance to purpura as a result of repeated injections of toxic doses of the purpura substance.

3. The serum of rabbits immunized with the purified purpura principle, with "S" and "R" strains of Pneumococcus or with cell extracts, autolysates or the nucleoprotein fraction of the organism does not confer upon white mice protection against purpura.

4. The purpura principle does not exist preformed in the cell, but is rather an autolytic derivative; since it is formed only when pneumococci undergo autolysis, and it is not found when the autolytic ferments are inactivated.

5. The purpura substance is associated with the proteose fraction of active pneumococcus extracts.

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CHANGES IN CARBON DIOXIDE TENSION AND HYDRO-GEN ION CONCENTRATION OF THE BLOOD FOLLOWING MULTIPLE PULMONARY EMBOLISM.

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INTRODUCTION.

In an effort to explain the cause of the extraordinarily accelerated respirations which result from multiple experimental embolism of the arterioles and capillaries of the lungs (1) it seemed important to observe what changes occurred in the blood in respect to its carbon dioxide tension and hydrogen ion concentration. The relative importance of these two factors in the control of respiration has been much discussed in the literature of late years, and opinion has emphasized first one, then the other. It is probable that both the pressure of carbon dioxide and the hydrogen ion concentration of the blood (or of the respiratory center itself) play an important rôle in the chemical regulation of respiration. The characteristic breathing of acidosis, due to the retention of non-volatile acids, such as is seen in diabetic coma, is hyperpnea, or a deep, labored, slow type of breathing. This, too, is the character of respiration which results from inhalation of gas mixtures rich in carbon dioxide (5 to 10 per cent). As far as we know, rapid and shallow breathing has not been definitely related to a retention of carbon dioxide, nor to an increase in the concentration of hydrogen ions. In at least one clinical condition (bronchopneumonia) (2), however, an increased content of carbon dioxide in the blood has been described, and we know that in this condition the respiratory rate may be elevated. That the accelerated respirations which we were attempting to explain resulted from an increased carbon dioxide tension or a fall in pH seemed unlikely from the outset. Still we considered it necessary to make actual measurements of these changes

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in relation to the changes in respirations before ruling them out as of importance. Especially was this true in view of the observation made by Dunn (3) that the inhalation of 10 per cent carbon dioxide by goats, breathing rapidly as the result of multiple pulmonary emboli, caused a reduction of the respiratory rate. In the light of our own observations, it seems probable that the cause of this reduction was probably an increased depth of respiration stimulated by the carbon dioxide in the inspired air. Such an increased depth would lead to an increase in alveolar oxygen tension and hence a decrease in the anoxemia. Anoxemia has been shown (1) to be a contributing factor to the rapid and shallow breathing resulting from multiple embolism of the pulmonary arterioles and capillaries, though not its cause.

EXPERIMENTAL.

The experimental procedure was a simple one. Dogs were anesthetized by the intravenous injection of barbital-sodium. A cannula was inserted into the left femoral artery, from which samples of blood were drawn for analysis, and into the right external jugular vein into which suspensions of potato starch cells were slowly injected. The blood was drawn without exposure to air and kept in sampling tubes over mercury. Analyses of carbon dioxide content were made in duplicate on the separated plasma or serum by the method of Van Slyke and Neill (4). The pH was determined on serum by the colorimetric method of Hastings and Sendroy (5). From these analytical data and from the following formula (6) the partial pressure of carbon dioxide expressed in mm. Hg was calculated, assuming pk' to be 6.115:

$$pCO_2 = \frac{[CO_2]}{0.031 \times (1 + 10^{pH - 6.115})}$$

where CO_2 content is given in terms of millimols per liter. In some experiments the tidal air and minute volume of pulmonary ventilation were measured by securing a tightly fitting mask to the dog's muzzle and collecting the expired air by means of flutter valves in a Tissot spirometer. In other experiments the respiratory rate was simply counted by observing the animal's thoracic movements. In most of the experiments, anoxemia was prevented by permitting the animal to breathe oxygen. It was soon learned that the rapid and shallow breathing which comes on after a certain volume of starch suspension has been injected, is usually associated with an increase in the carbon dioxide tension of the plasma and an accompanying fall in pH. But subsequent observations revealed the fact that these changes do not necessarily occur, and sometimes follow rather than precede, the onset of abnormally accelerated respirations.

To test the effect of the experimental conditions *per se*, without starch injection, on the carbon dioxide tension and hydrogen ion concentration, these measurements were made on a dog at approximately hourly intervals over a period of 3 hours. The results showed a fall in pCO_2 and a rise in pH, or a change in the opposite direction from that usually encountered following embolism. These facts are shown in Table I.

In the following three experiments (Nos. 4, 7 and 9) the accelerated respirations following starch embolism were accompanied by a marked

TABLE I.

Experiment 6. Effect of Barbital-Sodium Anesthesia on pCO_2 and pH of Dog's Blood and on Respiratory Rate.

Time	Respiratory rate per min.	CO₂ content	pCO_2	pH
		mM	*mm.Hg*	
2.47	38	23.37	46.20	7.30
4.00	34	22.02	38.20	7.36
5.00	36	21.67	36.80	7.37

TABLE II.

Experiment 4. Effect of Intravenous Starch Suspension Injection on Respiratory Rate, CO_2 Tension and pH of the Blood.

Time	Procedure	Respiratory rate per min.	CO₂ content	pCO_2	pH
			mM	*mm.Hg*	
4.04		16	21.17	47.50	7.23
4.12–4.32	Injection of starch suspension				
4.33		52			
4.35		51	22.28	60.68	7.15

increase in CO_2 tension (from 5 to 13 mm.) and a definite fall in pH (from 0.07 to 0.09). In each instance characteristic rapid and shallow breathing occurred, the rate increasing from an average of 18 to the minute to an average of 46, and the depth decreasing accordingly. The experimental data are brought out in Tables II to IV. It is to be noted that the actual change in CO_2 content expressed in millimols may be slight when the change in CO_2 tension is considerable.

Examination of Table III will bring out the following facts: In a

TABLE III.

Experiment 7. Effect of Intravenous Starch Suspension Injection on Respiratory Rate and Depth and on CO_2 Tension and pH of the Blood.

Time	Procedure	Respiratory rate per min.	Tidal air	Min. volume	$[CO_2]$	pCO_2	pH	Arterial blood		
								O_2 content	O_2 capacity	Per cent saturation
			cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	
12.12	Dog breathing room air	17	174	2.96	23.60	37.56	7.40	7.46	8.70	85.8
12.22	" "									
12.45–12.51	Injection of starch suspension									
12.53	Room air	46			25.10	40.82	7.39	7.26	8.96	81.1
1.00	" "	50	130	6.48	23.25	44.00	7.32	6.81	8.80	77.4
1.13	Dog breathing 95 per cent O_2									
1.20		50	123	6.18						
1.26					23.52	45.52	7.31	8.87	9.36	94.8

TABLE IV.

Experiment 9. Effect of Intravenous Starch Suspension Injection on Respiratory Rate and Depth and on CO_2 Tension and pH of the Blood.

Time	Procedure	Respiratory rate per min.	Tidal air	Min. volume	$[CO_2]$	pCO_2	pH	Arterial blood		
								O_2 content	O_2 capacity	Per cent saturation
			cc.	*liters*	*mM*	*mm. Hg*		*mM*	*mM*	
11.59	Breathing 95 per cent O_2 throughout experiment									
12.05		21	150	3.16	23.10	34.36	7.43			
12.17–12.26	Intravenous injection of starch suspension									
12.29		37	124	4.62	22.75	39.46	7.36	9.00	8.42	107

dog anesthetized with barbital-sodium, the intravenous injection of a suspension of potato starch grains resulted in an increase in respiratory rate from 17 to 50 per minute. This was associated with a decrease in tidal air amounting to 44 cc., or a 25 per cent decrease, and a resulting increase in the volume of pulmonary ventilation from 2.96 liters to 6.48 liters per minute. This change occurred while the animal was breathing room air and was accompanied by a drop in the oxygen saturation of the arterial blood from 85.8 per cent to 77.4 per cent. Accompanying this the pCO_2 rose from 37.56 mm. to 44.00 mm., with practically no change, however, in the CO_2 content of the serum as expressed in millimols. The hydrogen ion concentration, however, increased, as shown by the drop in pH from 7.40 to 7.32.

At this point oxygen was administered to the animal, with the result that the arterial oxygen saturation rose to 94.8 per cent, or the normal level, without, however, affecting the rate or depth of breathing, which still remained rapid and became even shallower than it had been. Nor was there a return of the pCO_2 or pH to the former level.

From these experiments it can be concluded that embolism due to intravenous starch suspension leads to rapid and shallow breathing, which is accompanied by a fall in the percentage oxygen saturation of the arterial blood, a rise in the pCO_2 and a fall in pH.

Restoring the blood to its normal oxygen saturation affects neither the respiratory rate or depth nor the carbon dioxide tension or hydrogen ion concentration. Similar results, as far as changes in pCO_2 and pH are concerned, were observed in Experiment 9, in which the animal was permitted to breathe oxygen throughout, so that the arterial blood remained completely saturated with oxygen. In this experiment the respiratory rate rose from 21 to 37 following starch injection, the tidal air falling from 150 cc. to 124 cc. A slight drop in the millimolecular concentration of CO_2 occurred, from 23.10 mM to 22.75 mM, but with a fall in pH from 7.43 to 7.36, the pCO_2 may be calculated to have risen by slightly more than 5 mm. These changes are brought out in Table IV.

Thus far no conclusions can be drawn as to whether the observed changes in pCO_2 and pH are the cause of the accelerated respirations or whether they result from the same condition which gives rise to rapid and shallow breathing.

To observe the effect on respiration of increasing the partial pressure of CO_2 in the serum without the disturbing influence of pathological changes in the lungs, such an increase was produced by permitting a dog to inhale from a Douglas bag containing a 10 per cent CO_2-90 per cent oxygen mixture. In this experiment (No. 12) in which the CO_2 tension of the serum was raised by CO_2 inhalation there was naturally an increase in the hydrogen ion concentration, both changes being consonant with those observed in the starch experiments. Instead of the development of rapid and shallow

TABLE V.

Experiment 12. Effect of Inhalation of 10 Per Cent CO_2-90 Per Cent O_2 Mixture on pCO_2, pH of the Blood and on Pulmonary Ventilation.

Time	Procedure	Respiratory rate per min.	Tidal air	Min. volume	[CO₂]	pCO₂	pH
			cc.	*liters*	*mM*	*mm.Hg*	
12.29	Breathing 95 per cent O_2	25.0	170	4.26			
12.35	" 95 " " "				26.21	40.76	7.41
12.38	" 95 " " "	30.4	194	4.90			
1.56	" 95 " " "	21.4	175	3.74			
2.03	" 95 " " "			·	26.28	42.82	7.39
2.05	" 95 " " "	21.4	180	3.86			
2.13–2.42	Breathing {10 per cent CO_2 {90 " " O_2						
2.30		42.0	390	16.42			
2.40					30.63	67.45	7.24
2.42		42.4	383	16.24			

breathing, however, the respirations, though they accelerated, more than doubled in depth. The average rate before CO_2 inhalation was 24, with a tidal air of 180 cc., compared to a rate of 42 after, with a tidal air of 383 cc. This was associated with a rise of CO_2 tension of nearly 25 mm., and a drop in pH from 7.40 to 7.24. These changes are exhibited in Table V.

This experiment (No. 12) is included simply for the purpose of demonstrating the well known effect of increasing the CO_2 tension and the hydrogen ion concentration, on the respirations. The significant difference from the starch effect is the marked increase in

depth. In many instances breathing high concentrations of CO_2 will increase the depth of respirations with little effect on rate.

Since, from the foregoing three experiments (Nos. 4, 7 and 9) it was impossible to say whether the changes in pCO_2 and pH antedated the onset of accelerated respirations, it was planned to draw the blood for analysis immediately after acceleration had begun. By this maneuver it was hoped to determine which change occurred first, *i.e.*, the change in CO_2 tension and pH or the change in rate and depth

TABLE VI.

Experiment 14. The Effect of Intravenous Starch Suspension Injection on Respiratory Rate and Depth, and on CO_2 Tension and pH of the Blood.

Time.	Procedure	Respiratory rate per min.	Tidal air	Min. volume	[CO₂]	pCO₂	pH
			cc.	liters	mm	mm.Hg	
11.43	Dog breathing 95 per cent O₂ throughout experiment	23.6	107	2.52			
11.49					24.40	51.48	7.27
11.52		23.2	120	2.78			
12.00–12.09	65 cc. 2 per cent starch suspension injected intravenously						
12.15		42.4	95	3.96			
12.21					23.58	49.75	7.27
12.24		47.6	89	4.24			
12.50					24.44	51.55	7.27
12.53		66.6	77	5.14			
1.09		73.0					

of respirations. It was found, in fact, that *accelerated respirations may occur before any measurable increase in CO_2 tension or hydrogen ion concentration.* From this the conclusion seems justified that the changes in CO_2 tension and pH are not the cause of rapid and shallow breathing which arises after embolism of the pulmonary arterioles and capillaries.

In Experiment 8 the respiratory rate, before starch injection, was 12 to the minute. At this time the pCO_2 was 66.95 mm. and the pH 7.23. After starch injection the rate had risen to 47 but the

$p\mathrm{CO_2}$ was 63.42 mm. and the pH 7.25. Similarly with Experiment 14, in the control period the respiratory rate was 23 per minute and the average tidal air 113 cc. Starch injection resulted in a rate of 48 to the minute with a decrease in depth to 89 cc. No significant change, however, had occurred in the $\mathrm{CO_2}$ tension or hydrogen ion concentration. The facts of this experiment (No. 14) are supplied in Table VI and in the appended protocol.

Protocol of Experiment 14. (See Table VI.)

Female, mongrel hound. Weight 12.2 kilos.

9.55. 3.36 gm. barbital-sodium, dissolved in 25 cc. distilled water, injected into the left leg vein.

10.05. Dog quiet, snoring. 10.30. Dog quiet, insensitive. Cannulated right jugular vein and right femoral artery.

11.00. Respirations 27. Pulse 205. Rectal temperature 38.4°C. Corneal reflex present.

11.08. Additional 5 cc. 5 per cent solution of barbital-sodium.

11.25. Respiratory mask adjusted to muzzle. 11.30. Breathing 95 per cent $\mathrm{O_2}$ from Douglas bag. 11.41. Connected with Tissot spirometer.

11.43. First respiratory period. Expired air collected for 5 minutes. Total volume 12.6 liters. 118 respirations.

11.49. 25 cc. bright red arterial blood drawn from right femoral artery.

11.51. Second respiratory period. Expired air collected for 5 minutes. Total volume 13.9 liters. 116 respirations.

12.00. Injection of 2 per cent starch suspension begun. Respirations continuously counted.

12.01. Respiratory rate 26.

12.03. Total of 50 cc. starch suspension injected.

12.04. Respiratory rate 24.

12.09. Total of 65 cc. starch suspension injected. Respiratory rate 35.

12.14. Third respiratory period. Expired air collected for 5 minutes. Total volume 19.8 liters. 211 respirations.

12.21. 25 cc. bright red arterial blood drawn from right femoral artery.

12.23. Fourth respiratory period. Expired air collected for 5 minutes. Total volume 21.2 liters. 238 respirations.

12.46. Respiratory rate 61 per minute.

12.50. 25 cc. bright red arterial blood drawn from right femoral artery.

12.52. Fifth respiratory period. Expired air collected for 5 minutes. Total volume 25.7 liters. 333 respirations.

1.00. Rectal temperature 38°C.

1.01. Respiratory rate 72.

1.09. Respiratory rate 73.

At this time the experiment was brought to a conclusion and the animal used for another purpose.

SUMMARY AND CONCLUSIONS.

1. The production of multiple emboli of the pulmonary capillaries and arterioles results in rapid and shallow breathing which may be associated with anoxemia, but is not dependent for its occurrence upon anoxemia.

2. Similarly there may occur an increase in the partial pressure of CO_2 in the blood as well as an increase in hydrogen ion concentration.

3. These changes must be regarded as the result of the impaired pulmonary function.

4. They are not, however, the cause of the rapid and shallow respirations, since the abnormal type of breathing may occur without the attendant blood changes.

5. The characteristic type of response to increase in CO_2 tension is an increased rather than a decreased depth of respiration.

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THE EFFECT OF MULTIPLE EMBOLI OF THE CAPILLARIES AND ARTERIOLES OF ONE LUNG.

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PLATE 23.

INTRODUCTION.

The striking fact (Dunn (1), Binger, Brow and Branch (2)) that obstruction to the pulmonary arterioles and capillaries leads to a marked disturbance of the respiratory mechanism with the development of rapid and shallow respirations, and an increase in the minute volume of pulmonary ventilation, presents a pathological state which we have attempted to analyze further. Interest in this effect lies partly in its clinical application to such conditions as lobar pneumonia in which accelerated respirations are an important physical sign; and partly in the opportunity for investigating the difficult problem of respiratory rhythmicity and some of the factors influencing it. The reasons for thinking that an analogy exists between the rapid breathing due to multiple emboli of the arterioles and capillaries and the rapid breathing occurring in lobar pneumonia are several. In the first place in both conditions rapid breathing may occur independently of anoxemia. In the animal with multiple emboli this fact has been experimentally shown (2). And we have frequently observed patients with pneumonia continuing to breathe at the rate of 40 or more to the minute even after oxygen want has been relieved by oxygen administration. The occasional persistence of rapid respirations after crisis, when pulse and temperature have returned to normal, has suggested that the stimulus for accelerated respirations may be a local one resulting from the pulmonary lesion. A probable occurrence of capillary fibrinous thrombi has been described in pneumonic lungs by Kline and Winternitz (3), and that obstruction to the circulation may

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occur in the consolidated lobe is known. This last fact again suggested a possible similarity in causes operating to produce similar effects.

Moreover, it has been found in experimental pneumonia produced in dogs by intratracheal insufflation of cultures of B. *friedlænderi* (Porter and Newburgh (4)) that the dyspnea which resulted could be checked by sectioning the vagi or blocking vagal impulses with cocaine. A similar slowing of accelerated respirations has been demonstrated for the tachypnea resulting from multiple experimental emboli. In this condition either vagal section (1) or vagal freezing (2) immediately brings about slow, deep breathing.

Quite aside from its immediate application to disease, a study of changes in respiratory rate has appeared important to us from the point of view of the nervous control of respiration. Of this subject not much is known. We know that the so called respiratory center sends out impulses which vary in strength and frequency, according to certain changes, physical and chemical, occurring either in the blood stream or in the center itself. And we know that the character of breathing, *i.e.* rate and depth, is influenced in some manner by impulses travelling along the vagus nerves (Le Gallois (5), Herɪng and Breuer (6), Head (7)). That these impulses are of a centripetal nature may be assumed from the studies of Einthoven (8) in which he demonstrated electrical changes in the thoracic end of the cut vagus occurring synchronously with changes in distension of the lungs. Whether there are centrifugal vagal impulses important to the control of respiration is not known. The vagus nerves in the dog, more than in the cat and rabbit, are not pure nerves, but are colonies containing sympathetic and depressor fibers as well. These nerves supply both heart and lungs. Cutting and freezing experiments are therefore necessarily varied in their effects and difficult to interpret.

The perhaps undue emphasis which we have placed on respiratory rate rather than on the minute volume of pulmonary ventilation lies in our interest in the nervous mechanism, where change in rate must represent a reversal in impulse occurring either peripherally in the breathing apparatus or in the respiratory center.

We must again emphasize the great difficulties introduced by the use of anesthetics. One is indeed caught between the horns of a

dilemma. To study the respiration in animals without an anesthetic is often impossible when concerned with changes involving the circulation in the lungs. We have previously (2) alluded to the effects of emotional and other adventitious stimuli which may confuse an experimental procedure. And yet equally confusing may be the sometimes excitatory, sometimes depressing influences of the anesthetic used to obviate the other effects. We have varied our technique, using luminal sodium by stomach tube, or barbital-sodium given intravenously or again ether with sterile technique and survival, in animals previously trained to lie still and breathe quietly. In the interpretation of results we have had constantly to keep in mind the effects of anesthetics.

The characteristic gross and microscopic pathology of the lungs of dogs in which multiple emboli of arterioles and capillaries have been produced by injection of suspensions of starch cells is congestion, edema and atelectasis. This is associated with a diminution in lung volume as determined by a measurement of the functional residual air (2). It seemed not improbable that these changes resulted in a decrease in elasticity of pulmonary tissue producing a shallow tidal air and thus, through the mechanism of the Hering-Breuer reflex, a rapid respiratory rate. Could the phenomenon be reproduced by causing similar lesions in one lung? If so, this would tend to establish more clearly the analogy with rapid and shallow breathing as it occurs in pneumonia. Experiments were planned with an eye to answering this question. It must be stated in advance that because of the technical difficulties involved and the necessary operative trauma the answers were not as unequivocal as was hoped.

EXPERIMENTAL.

Experiment 1. Effect of the Injection of Starch Suspension into One Lung.—
A female dog, weighing 7 kilos, was given by slow intravenous injection 2.50 gm. barbital-sodium dissolved in distilled water, or 0.34 gm. of the drug per kilo of body weight. Within ½ hour the dog was quiet and relaxed, and breathing regularly at the rate of 13 respirations per minute. The animal was then intubated and artificial respiration started by the intratracheal insufflation of an interrupted current of air. With the dog lying on its right side the thorax was opened by incising the skin, muscles and pleura in the 4th left intercostal space. The ribs were separated by mechanical retractors, and with very little blunt dis-

section the artery to the left lung was freed.   By a method to be described below a suspension of starch grains (sufficient ordinarily to produce rapid and shallow breathing when injected intravenously) was injected directly into the artery supplying the left lung.   Thereupon the animal's thorax was closed by approximating the 4th and 5th ribs by three stout ligatures, care being taken to distend the lungs and drive all air out of the pleural space before closing the chest.   The muscle and skin layers were next repaired by suturing, the dog turned on its back and allowed to breathe spontaneously after removing the tracheal tube.   To avoid the complications of anoxemia a stream of oxygen was blown through a funnel placed over the dog's muzzle.   In this animal the preoperative respiratory rate varied from 16 to 20 breaths per minute.   The postoperative rate, after the temporary acceleration had subsided, varied from 24 to 21.   Following a steady respiratory rate at this level for 25 minutes the dog was killed by the intravenous injection of 20 cc. of a saturated solution of magnesium sulfate, and autopsied.   The abdominal cavity and its viscera were normal.   On opening the thorax the left lung, containing the starch grains, was mottled and paler than the right, which appeared normal.   Frozen sections of the lungs, stained with Lugol's solution, showed the four lobes of the right lung to be free from starch grains, whereas in the small vessels of the left lung, especially in the upper and ventral lobes, they were thickly distributed.

From this experiment the following tentative conclusion can be drawn: Embolism of the capillaries and arterioles of one lung (the left) does not result in rapid and shallow breathing, when the other lung is functioning normally.   The phenomenon is therefore not of an irritative nature due, solely, to the presence of the embolic material in one lung.

There is one important criticism to this experiment which obviously makes it inconclusive, namely: Was there actually enough embolic material introduced into the left lung to produce rapid and shallow breathing?   It has repeatedly been observed that with intravenous starch suspension injections into the intact, unoperated animal a certain volume of suspension is necessary before accelerated respirations start.   This fact was interpreted as indicating that the phenomenon was in some manner related to the degree of obstruction to the pulmonary circulation rather than the result of irritative, local stimuli set up in the lungs.

Method of Injecting Starch Suspensions Directly into the Artery Supplying One Lung.

As this method may prove to be of use to others it seems worth describing in detail. The method has the advantage of allowing the injection to proceed without clamping the artery or interfering with the circulation in the lung. The injection apparatus consists of a graduated burette to which about 10 inches of rubber tubing are attached. The tubing passes through a du Noüy* pump, which operates by rotating a wheel, designed to compress and decompress alternately
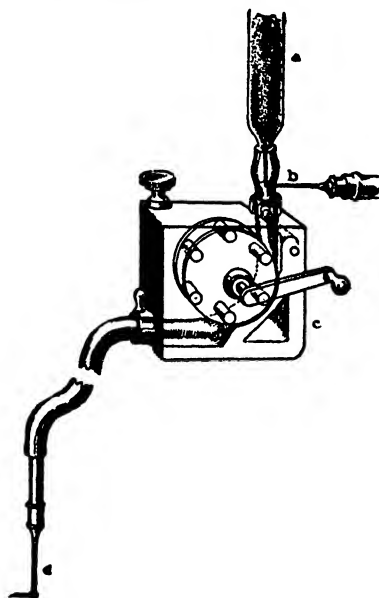
TEXT-FIG. 1. Apparatus for injecting suspensions into the pulmonary artery or its branches. *a*, burette; *b*, hollow needle through which a stream of air is blown to keep suspended particles from settling; *c*, du Noüy pump; *d*, bent needle with ball of solder in place.

the rubber tubing, thereby ejecting the fluid which enters the tubing from the burette. The speed and force of injection can be varied at will by varying the number of rotations of the wheel.

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* This very useful pump, which was devised by Dr. du Noüy, of The Rockefeller Institute, has not previously been described in the literature. It was manufactured by the Central Scientific Company. We are indebted to Dr. du Noüy for its use.

The distal end of the rubber tube connects with a No. 21 gauge Lüer needle 3 cm. long. 1 cm. from its point this needle has been bent at right angles by heating to a dull red, bending with a pair of nippers and then retempering. A small ball of solder is fused on to the needle at the right angle bend. Before thrusting the needle point through the arterial wall a small piece of muscle is "baited" on to the needle in much the same manner as baiting a fish hook. The ball of solder keeps the fragment of muscle from sliding around the bend and up the shank of the needle. This muscle fragment serves the double function of a flap valve over the puncture wound and of supplying tissue extracts which act as coagulants. With this equipment the arterial wall can be punctured with little hemorrhage and the needle kept in place for 15 to 20 minutes or more, without interfering with the flow of blood through the vessel. On removing the needle, the bleeding which usually occurs at the puncture wound can be controlled by the application of a fragment of muscle and cotton pledgets moistened in warm saline. Text-fig. 1 illustrates the burette, pump and needle used for injecting suspensions into the pulmonary artery or its branches.

Experiment 2.—An important corollary to the foregoing experiment, and one free from the criticism applicable to it, is the following, in which a dog, similarly anesthetized with barbital-sodium, was prepared for the experiment by ligating the left branch of the pulmonary artery. After the thorax had been closed, the animal was allowed to breathe oxygen. The respiratory rate at this time was 10 to the minute. A starch suspension of the same strength as in the previous experiment was then introduced into the circulation through a cannula in the right jugular vein. Since the left branch of the pulmonary artery had been ligated, all the starch entered the vessels of the right lung. The result was rapid and shallow breathing, the rate reaching a level of 58 to 60 per minute, where it remained for 1 hour before the animal was killed. The absence of anoxemia, as a contributing cause to the rapid breathing, was established by analysis of the dog's arterial blood at the close of the experiment, the percentage oxygen saturation being 100.

At this point, the tentative conclusion can be drawn that emboli in the capillaries and arterioles of one lung do not produce rapid and shallow breathing when the circulation through the other lung is normal. Or, in other words, the presence of embolic material, such as starch grains in one lung, does not excite this change in respirations. When, however, the circulation to the other lung is cut off by ligating its artery, emboli of capillaries and arterioles of one lung do produce rapid and shallow breathing in spite of the absence of oxygen want. Under the conditions of the experiment, the "embolized" lung adequately satisfied the animal's oxygen needs, but, even so, respirations assumed a definitely abnormal and accelerated character.

In an effort to surmount our own objections to the first experiment of this pair (Experiment 1), a new approach was devised.

A method was employed by which the left branch of the pulmonary artery could be occluded after the thorax had been closed and while the animal was breathing naturally. The occlusion was accomplished by inflating a pneumatic rubber cuff which had been previously placed about the left branch of the pulmonary artery. The method is given in detail in another paper in this *Journal* (9). Starch suspensions were then injected intravenously so that the emboli lodged in the vessels of the right lung. The left branch of the pulmonary artery was then released. By so doing, blood was allowed to circulate through the left lung after emboli, sufficient to produce rapid and shallow breathing, had lodged in the right lung. Under these conditions, does reestablishing the circulation through the left lung restore the respiratory rate to its original level? It should be stated that in these experiments too, anoxemia was prevented by oxygen inhalation.

The results of this series of experiments were variable. In some, reestablishing the circulation to the left lung (after production of rapid and shallow breathing by intravenous injection of starch granules which had lodged in the capillaries of the right lung) was accompanied by a definite slowing of respiratory rate. In others, the rate remained accelerated. In only one experiment (No. 3), was the respiratory rate restored to its slow, preoperative level.

In this experiment, the dog breathed at the rate of 16 per minute before it was operated upon, and the arterial blood was 97 per cent saturated with oxygen. The thorax was then opened and a pneumatic cuff placed about the left branch of the pulmonary artery. After closing the thorax, and allowing the animal to breathe naturally, the rubber cuff was inflated through a tube which projected through the chest wall. Inflation of the cuff completely shut off the flow of blood to the left lung. This resulted in no increase in respiratory rate. Indeed, at this stage of the experiment, the dog was breathing 13 times per minute. A starch suspension was thereupon injected intravenously, with the result that the respirations became shallow and accelerated to the rate of 61 per minute, despite the fact that the arterial blood was still 95 per cent saturated. The obstruction to the left branch of the pulmonary artery was next released by allowing the cuff to deflate, with the result that within 4 minutes the respiratory rate had dropped to 11 per minute.

This experiment is a clean-cut one and is more susceptible of interpretation than the others of this series. The dog breathed normally with only one lung, the right. When small emboli were introduced into the right lung, the respiratory rate accelerated to nearly 5 times

its previous rate. When, however, circulation was reestablished in the left lung, the respiratory rate returned to normal. Clearly, then, from this experiment too, one could conclude that emboli in the capillaries of one lung alone (the right) do not produce rapid and shallow breathing, unless the other lung is thrown out of the circulation, or, (as is suggested below), otherwise structurally damaged. That this last may account for the variable results referred to above is suggested by the following observations. In several experiments, the left lung, which had been temporarily deprived of its arterial blood, was, at autopsy, found to be bright red in color, instead of the normal salmon-pink. We know that depriving an organ of its blood supply may result in hyperemia from dilated capillaries, when the blood is again admitted (10). It is our opinion, though this fact is not definitely established, that in those animals in which accelerated respirations persisted after reestablishing the circulation to the left lung, some such capillary damage had been produced.

Experimental evidence for this statement is supplied by histological studies of the left lungs of Dogs 3 and 4. In Dog 3 (the experiment cited above in which the rate returned to normal on release of the cuff) the microscopic anatomy of the left lung is essentially normal. In Dog 4, however, in which the left lung, at autopsy, was noted to be bright red and granular in appearance, the respiratory rate remained at 61 per minute even after release of the obstruction to its artery. Here the microscopic picture is definitely abnormal, the thickness and distortion of capillaries being plainly visible. We reproduce photomicrographs of two typical regions of the left lungs of these two dogs which show clearly this difference in them (Figs. 1 and 2).

It should be stated that the variable behavior of different animals could not be correlated with variations in the percentage of oxygen saturation of the arterial blood, nor in the CO_2 tension or pH of the blood (11). Since this is true, we have not considered it worth while to present these data in this publication.

DISCUSSION.

When a suspension of potato starch grains is intravenously injected into a dog, the grains lodge in the finer arterioles and capillaries of the lungs. No change in the dog's respirations occurs until a certain, somewhat variable (in different animals) dose has been given. The

dog then develops rapid and shallow breathing, the respiratory rate often reaching 60 or more per minute. This phenomenon has interested us, partly because of an apparent analogy to the accelerated respirations seen clinically in cases of lobar pneumonia, partly because we believed that an understanding of this condition might throw some light on the problem of the rhythmicity of respiratory movements, and in particular upon the so called Hering-Breuer reflex.

Previous work has not revealed the cause of this abnormal type of breathing. It has not been correlated with (1) changes in arterial or venous blood pressures, (2) changes in percentage saturation of the arterial blood with oxygen, (3) changes in carbon dioxide tension or pH of the blood. It was found, however, to be associated with a reduction in lung volume, as expressed by measurement of the so called functional residual air, or that volume of air remaining in the lungs at the end of a quiet, normal expiration. And it was found that the characteristic pathological picture associated with this abnormal type of breathing was congestion, atelectasis and edema of the lungs.

The present study was undertaken to discover whether similar emboli introduced into one lung would result in a similar change in breathing. To accomplish this, that is, introduction of emboli into one lung, we had to resort, not only to the use of anesthetics, but to difficult and drastic operative procedures. Such procedures, of course, have drawbacks, and we have previously dwelt on the care necessary in interpreting results in the face of them.

Experimental evidence here presented appears to us to justify the conclusion that emboli lodged in the arterioles and capillaries of one lung produce an accelerated type of breathing only when the other lung has been excluded from the circulation by ligating or clamping its artery, or when the capillaries of the other lung have been abnormally distended by depriving them of blood for a period and then again admitting the blood.

The phenomenon would, therefore, appear to be in some manner related to the condition of diminution of the pulmonary vascular bed, to congestion in the lung or to resistance to the flow of blood through the lungs.

SUMMARY AND CONCLUSIONS.

1. Injection of a suspension of potato starch cells into the left branch of the pulmonary artery, in quantity sufficient ordinarily to give rise to markedly accelerated respirations, resulted in no change in respiratory rate.

2. A method for injecting substances into the pulmonary artery or its branches without interfering with the blood flow to the lungs has been described.

3. Injection of similar material into one lung when the other is excluded from the circulation either by ligation or by temporary clamping does give rise to rapid and shallow breathing (from a rate of 10 to 15 per minute to one of 60 or over) identical in character to that brought about by introducing emboli into both lungs.

4. A method for clamping and releasing the pulmonary artery or its branches in a dog breathing normally with closed thorax has been devised.   This is described in detail in another paper.

5. After rapid breathing has been initiated by the effect of emboli lodged in the arterioles and capillaries of the right lung, reestablishing the circulation in the other lung by releasing the clamp on its artery may or may not restore the respiratory rate to its original, normal level.

6. This discrepancy in results has not been correlated with any difference in oxygen saturation of the arterial blood, or in carbon dioxide tension or pH of its plasma.

7. It is, however, believed to be related to the gross and microscopic anatomy of the lung of which the artery has been temporarily clamped. Photomicrographs are published, showing in one dog (No. 3), in which the respiratory rate returned to normal, a normal histological picture of the left lung, and in another dog (No. 4), in which the rate remained rapid after release of the clamp, a picture characterized by congestion and dilatation of arterioles and capillaries.

8. The fact that accelerated respirations result from emboli in the pulmonary capillaries and arterioles only after a certain quantity of material has been introduced, and the fact that emboli in one lung do not occasion accelerated respirations unless the circulation through the other lung is occluded or abnormal, leads us to the conclusion that

the phenomenon is not an irritative stimulus due to foreign bodies, but is in some manner related to (*a*) diminution of the pulmonary vascular bed, (*b*) resistance to the blood flow through the lungs or (*c*) congestion or dilatation of the arterioles and capillaries of the lungs.

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EXPLANATION OF PLATE 23.

FIGS. 1 and 2. Photomicrographs of sections of lungs from Experiments 3 and 4. × 130.

FIG. 1. Experiment 3. Section from left upper lobe. The histological picture is essentially normal.

FIG. 2. Experiment 4. Section from left upper lobe. The picture shows marked engorgement and tortuosity of alveolar capillaries.

Both preparations were made with similar technique, the dogs being killed by the intravenous injection of a saturated solution of magnesium sulfate. The lungs were fixed by immersion in Helly's fluid, after they had been distended *in situ* by the intratracheal injection of about 1 liter of Helly's fluid.

PLATE 23.
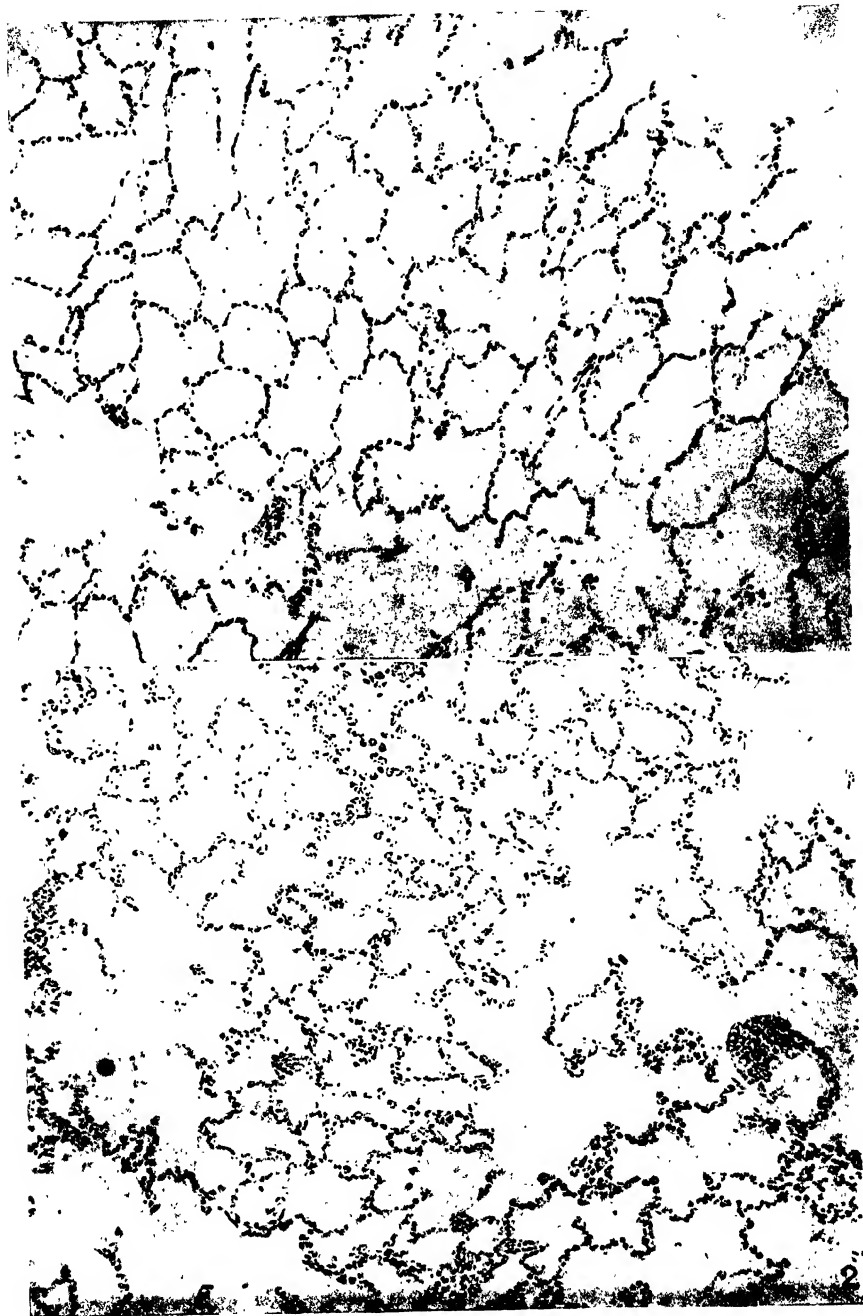
OBSERVATIONS ON RESISTANCE TO THE FLOW OF BLOOD TO AND FROM THE LUNGS.

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PLATES 24 AND 25.

INTRODUCTION.

Previous studies (1–4) have led us to the view that the cause of rapid and shallow breathing which results from multiple emboli in the arterioles and capillaries of the lungs must be sought for in the secondary vascular changes following embolism. Neither the presence of the embolic material *per se* nor the chemical changes found in the blood can be regarded as the direct cause of the modified type of breathing. The phenomenon appears to be related in some manner to the diminution of the pulmonary vascular bed, to the resistance to the flow of blood through the lungs or to the state of congestion and edema of the lungs. It has already been shown (2) that obstruction to the larger branches of the pulmonary artery by the intravenous injection of seeds of various sizes results in a condition quite different from that due to capillary obstruction. The functional abnormalities in breathing thus produced were found to result wholly from the condition of oxygen want. That blocking the capillary bed should have a different effect from blocking some of the larger branches of the pulmonary artery is not hard to understand. The first might be described as an "effective blockade" in which the blood passing through the lesser circulation must necessarily meet resistance. In blocking some of the larger branches, however, the blood has an alternative route to follow, and such resistance may not be encountered.

The probability that resistance to flow of blood through the lungs would result in heightened pressure in the pulmonary artery and perhaps the right heart was borne out by the work of Haggart and Walker

(5) and of Wiggers (6).   It occurred to us that such pressure changes might result in stretching of the walls of the pulmonary artery or of the right ventricle and that this might induce afferent stimuli which could occasion reflex changes in respirations.   No experimental evidence for this assumption, however, can be adduced from this work, and the results here published, though perhaps negative from the point of view of explaining the origin of rapid and shallow breathing, are of themselves of interest, we believe, and have helped us with an understanding of the problem.

<div align="center">EXPERIMENTAL.</div>

Dilatation of the Pulmonary Artery and Right Ventricle Following Obstruction to the Arterioles and Capillaries of the Lungs.

Without indulging in the artificial conditions necessarily prevailing in cardiac oncometry an effort was made to determine what changes occurred in the state of dilatation of the pulmonary artery and right ventricle after obstructing the flow of blood through the lungs by the intravenous injection of a suspension of potato starch grains.   This was done by careful x-ray photographs made of the heart during injection of starch suspensions.   A comparison of the photographs made under uniform conditions before and after the onset of rapid and shallow breathing shows a marked area of bulging at the base of the heart on the left side and a flaring out of the shadow cast by the right side of the heart.   This was clearly shown in two experiments (Nos. 1 and 2).   The x-ray photographs in Experiment 1 taken before and after the onset of rapid breathing following starch injection are reproduced in Fig. 1.   The bulge at the left side of the base is plainly to be seen, as well as the flare in the shadow cast by the right side of the heart. At the time of the first picture the dog was breathing at the rate of 16 per minute; at the time of the second the respiratory rate was 64. The area of the cardiac shadow, measured by a planimeter, averaged 43.4 sq. cm. before the injection of starch as compared with 48.4 sq. cm. after.   And the maximum transverse diameter of the cardiac shadow before embolism was 6 cm. as compared with 6.7 cm. after.

Like results were obtained in Experiment 2, in which x-ray photographs showed similar bulging at the left base of the heart and flaring

of the right border. By taking a series of x-ray plates during the experiment it was shown that these changes occurred synchronously with the acceleration of respirations. These facts are made plain by the two curves shown in Text-fig. 1 in which the lower represents
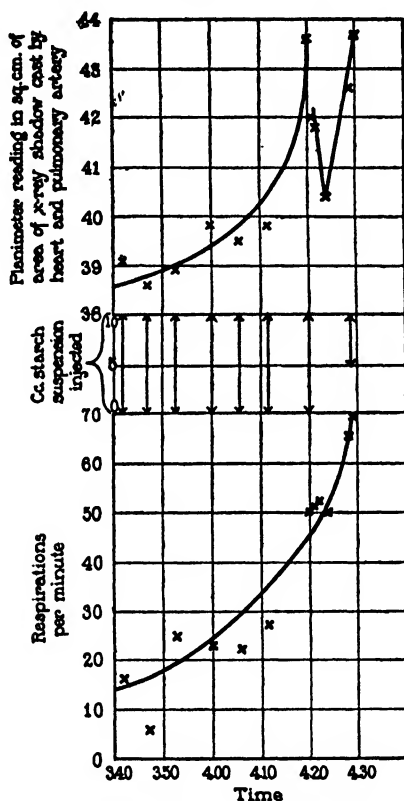
TEXT-FIG. 1. Lower curve: Ordinates, respirations per minute; abscissæ, time. Upper curve: Ordinates, area in sq. cm. as measured by a planimeter of x-ray shadow cast by the heart and pulmonary artery; abscissæ as in lower curve. The length of each arrow represents the volume of starch suspension injected at the time specified in the abscissæ.

respiratory rate per minute at the times specified in the abscissæ, and the upper, the area of the cardiac shadow in sq. cm. as measured by a planimeter. The arrows represent the times at which suspensions of starch were intravenously injected. The two curves will be

seen to vary synchronously and in the same direction.   The break at the end of the upper curve may be regarded as a temporary recovery of muscular tone overcome by the injection of an additional 5 cc. of starch suspension.

The planimeter measurements recorded here and in Experiment 1 were made by tracing the whole dense shadow cast by the heart, including the bulge at the left base which was suspected of being caused by the dilated pulmonary artery.   By tracing the ventricular shadow alone from arbitrary points after the method of Levy (7) and Stewart (8), it was found that the increase in area was far less marked than it was when the bulge was included in the tracing.   Direct inspection of the photographs shows the heart after embolism to be wider and shorter than before.

By carefully splitting the sternum without loss of blood in a dog* breathing at the rate of 56 per minute following starch embolism (Experiment 3) and maintaining the same respiratory rate by interrupted, intratracheal insufflation, after the chest had been opened, it was shown beyond doubt that the shadow of the bulge at the left base of the heart previously alluded to was cast by a greatly dilated pulmonary artery.   This structure stood out a tense, bulging, sausage-like mass, three or four times its normal size.

The experimental facts thus far presented, we believe, prove that *obstruction to the capillaries and arterioles of the lungs results in an increase in the area of the x-ray shadow cast by the heart, and that this increase is the result partly of a widening of the transverse diameter of the ventricles, but chiefly of a marked bulging of the pulmonary artery.   The changes in the heart and pulmonary artery were shown to occur synchronously with the acceleration of respiratory rate.*

The hypothesis on which we proceeded, and which was found to be faulty, was that the dilated pulmonary artery sent out impulses which reflexly accelerated respirations.   Though the results of these experiments are in a sense negative, by eliminating false causes, they largely clarify our problem.

Experiments were devised with an eye to obstructing the blood flow to the lungs proximal to the pulmonary parenchyma itself so that

* The animal was anesthetized with barbital-sodium.

any changes in breathing could be attributed wholly to the cardio-vascular effect, unclouded by pathological changes in the lungs. Two methods were used to accomplish this end: (1) the exposed heart method of Drinker; (2) a new method which will be described in detail below.

Experiments with the Drinker Exposed Heart Preparation.

By the technique devised by Drinker (9) the heart of a cat was exposed through a window cut into the sternum and the thorax closed by stitching the split pericardium to the edges of the window. The heart thus everted and supported on its pericardial sling, could be observed and its great vessels manipulated at will while the animal breathed without the aid of artificial respiration. To obstruct the flow of blood through the pulmonary artery, or one of its branches, a clamp and ligature were placed about it, after the method described by Haggart and Walker (5), in such a manner that progressive tighten-ing of the ligature resulted in progressive diminution of the calibre of the vessel until it was completely occluded.

This experiment was performed on two cats anesthetized with barbital-sodium. In one of them (No. 4) the clamp was placed on the left branch of the pulmonary artery, in the other (No. 5) on the main trunk of the artery. Since the results of these two experiments are in complete agreement with those of Haggart and Walker (5) they will not be given in detail. In Experiment 4, in which the left branch of the pulmonary artery was suddenly occluded, no change occurred in the animal's respirations. In Experiment 5, in which the main trunk of the pulmonary artery was gradually compressed, no change in respiratory rate or depth occurred until a point was reached when respirations ceased entirely.

Because all of our previous studies on the effect of pulmonary embolism on breathing had been made on dogs, it was determined to repeat the last two experiments on dogs instead of cats. This, how-ever, brought us face to face with a real difficulty. The Drinker ex-posed heart preparation, though admirable in the cat, cannot be used satisfactorily in dogs because of the depth of the dog's thoracic cavity, and the difficulty to effect a tight closure between the pericardial edges and the chest wall. Moreover, it seemed desirable to devise a

method in which the thoracic cavity was not restricted in size and one which could be used, if need be, in experiments made without anesthesia, on animals previously prepared by sterile, survival operations.

The method finally achieved was the following: A thin walled, flat rubber bag measuring 1.8 × 5.5 cm. communicating with a 30 cm. length of rather heavy walled rubber tubing (3 mm. in cross-section with a 2 mm. bore) was passed under the previously exposed and freed pulmonary artery.* By surrounding this rubber bag or cuff with a stocking of closely woven silk, to the four corners of which ligatures were attached, the rubber bag could be fastened in position about the vessel. The silk covering prevented overdistension of the bag on inflating it, and assured a uniform compression on the vessel, similar to that produced by the ordinary blood pressure cuff. Inflation of the bag was made through the patent end of the rubber tubing. The pressure in the cuff could be accurately controlled by the use of a mercury manometer. Fig. 2 illustrates the bag in place before the ligatures were fastened. When this is done, the bag forms a closely fitting cuff surrounding the vessel wall. Inflation immediately compresses the vessel. It was found that a pressure of 150 mm. Hg was more than sufficient to obliterate the lumen of the artery. As has been said, the method could be used in survival experiments in which the pulmonary artery or one of its branches could be occluded without the use of anesthetics, and after the effect of operative trauma had subsided. Fig. 3 is a photograph of such a survival experiment (No. 6) to be referred to below. The rubber tube, projecting through the chest wall, through which the bag is inflated, is shown in the picture.

Effect on Respiration of Compression of the Left Branch of the Pulmonary Artery in the Anesthetized Dog.—Dogs anesthetized by the intravenous injection of barbital-sodium were operated on, with the aid of artificial respiration, an incision into the pleural cavity being made through the 4th left interspace. Spreading the 4th and 5th ribs gave free access to the left branch of the pulmonary artery after the upper and middle lobes of the left lung had been carefully retracted downward and packed out of the field. The left branch of the pulmonary artery was surrounded by the pneumatic cuff described above and the chest wall closed by approximating the spread ribs with three stout ligatures after proper distention of the lungs. In closing the thorax, the free end of the rubber tube which communicates with the cuff was permitted to project through the chest wall.

* When one of the two main branches of the pulmonary artery was compressed, a somewhat smaller bag was used, *viz.*, 0.8 × 4.2 cm.

The effect on breathing of compressing the left branch of the pulmonary artery was noted in a series of experiments. The results obtained are similar to the cat experiment (No. 4) cited above. Blocking the circulation to the left lung produces essentially no immediate (2 minutes to 2 hours) effect on respiratory rate. Five animals showed a slight drop in rate, -1, -3, -3, -1 and -2, respectively. One animal showed no change. Eight animals showed a slight increase in rate, $+2$, $+6$, $+1$, $+5$, $+1$, $+3$ and $+1$. Whereas the average postoperative control rate was 22.5 per minute, the average rate after compression of the pulmonary artery was 24.2 The periods of compression, during which the respiratory rates were observed, varied from 2 minutes to 2 hours. Anoxemia was prevented from arising in these experiments by allowing the animals to inhale oxygen.

From these observations it can be definitely concluded that *restriction of the pulmonary vascular bed by nearly half does not result in rapid and shallow breathing*.

In two of this series of experiments (Nos. 7 and 8), the carbon dioxide tension and hydrogen ion concentration of the serum were studied before and after excluding the left lung from the circulation.

In Experiment 7 there was an increase of nearly 10 mm. Hg in $p\mathrm{CO_2}$ with a corresponding drop in pH from 7.34 to 7.27. In Experiment 8, however, the $p\mathrm{CO_2}$ fell from 54.05 mm. Hg, before clamping the left branch of the pulmonary artery, to 50.35 mm. Hg after, and the pH rose from 7.29 to 7.32. Tables I and II give the analytical data of these two experiments.

No constant effect, therefore, on the $\mathrm{CO_2}$ tension or pH of the serum is to be anticipated from suddenly shutting off the circulation to the left lung.

Effect on Respiration of Gradual Compression of the Main Trunk of the Pulmonary Artery in the Surviving Unanesthetized Dog. Protocol of Dog 6.—(See Fig. 3.) February 3, 1926, a white and black, male setter, weighing 14.5 kilos, was given ether by cone after a preliminary injection of morphine. When the dog was fully relaxed a rubber tube was inserted into the trachea. Artificial respiration and anesthesia were maintained by forcing an air-ether mixture intermittently into the lungs at a pressure of 20 mm. Hg. The dog was placed on its right side, with fore legs extended. Thorax was shaved and skin scrubbed with soap, water and alcohol. The chest was opened by resecting the 5th rib from its costochondral junction laterally for a distance of about 3 inches. By a self-

retaining mechanical retractor the edges of the wound were held apart and the left lung was retracted from the field by a cotton pad moistened with warm, physiological saline solution. A vertical incision was then made in the anterior surface of the pericardium directly over the pulmonary artery, from the point at which

TABLE I.

Experiment 7.

Time	Procedure	Respirations	[CO₂]	pCO₂	pH
		per min.	*mM*	*mm.*	
1.41	Postoperative control period. Cuff in place but deflated. Dog breathing oxygen throughout experiment	20	27.56	50.40	7.34
1.46	Cuff inflated to 150 mm. Hg				
2.10		26			
2.40		24			
3.25		26	28.45	59.60	7.27

TABLE II.

Experiment 8.

Time	Procedure	Respirations	[CO₂]	pCO₂	pH	O₂ capacity	O₂ content	O₂ saturation
		per min.	*mM*	*mm.*		*mM*	*mM*	*per cent*
2.14	Postoperative control period. Cuff in place but deflated. Dog breathing oxygen throughout experiment	24	26.76	54.05	7.29	8.97	8.88	99
2.17	Cuff on left branch of pulmonary artery inflated to 150 mm. Hg							
2.52		25	26.71	50.35	7.32	9.02	9.10	101
2.52	Cuff deflated							
3.24		25	26.63	49.35	7.33	9.17	9.06	98.8

the pericardium is reflected from the great vessels downward for a distance of 2 inches.

The edges of the pericardium were retracted by temporary ligatures. The pulmonary artery was freed from the aorta by blunt dissection and the cuff tied snugly in place. After approximating the edges of the pericardium, the wound

was closed in layers, the tube connecting with the cuff being led through the incision as far as the platysma muscle, then beneath this muscle, to make its exit through the skin at a point on the dorsolateral aspect of the chest, 3 inches distant from the wound. This was an added precaution taken against the possibility of infection working its way in from the outside. Silk ligatures and sutures were used throughout.

The dog made a splendid recovery and on February 9, 1926, appeared well and acted normally. The only sign of infection was a slight, odorless discharge, which could be expressed about the tube at the point of its exit in the skin. Weight on this date, 13 kilos. The dog had shown a very decided disinclination toward food, but there had been some improvement in this respect over the immediate postoperative period.

February 9, 1926. On four separate occasions the tension in the cuff was gradually raised to the point of producing respiratory failure, without any increase in respiratory rate. Each break was characterized by identically the same symptoms. The dog would suddenly become restless, lift up its head and then begin to whine. This was immediately followed by muscle rigidity, and the respirations, which had become irregular, abruptly ceased. Immediate deflation of the cuff restored the animal to its normal state within a few seconds. The results obtained during the first and second compressions on this date are shown in Tables III and IV.

Similar results were obtained in another dog (No. 10) of this series. In this experiment it was observed at operation that inflation of the rubber cuff resulted in a marked dilatation of that portion of the pulmonary artery proximal to the cuff.

The conclusion can now be definitely drawn that *resistance to the flow of blood to the lungs obtained by sudden or gradual compression of the pulmonary artery, in both the cat and the dog, though it results in dilatation of the pulmonary artery and of the right chambers of the heart, does not give rise to rapid and shallow breathing as do multiple emboli of the pulmonary capillaries and arterioles, but produces practically no change in respirations until syncope and respiratory failure suddenly occur.* From this it is likewise concluded that the markedly accelerated and shallow respirations following embolism of the pulmonary capillaries and arterioles are not the result of a reflex stimulus arising in the dilated heart or pulmonary artery.

Effect of Gradual Compression of the Pulmonary Veins on the Heart and Respirations.—It remained to discover what effect impeding the return of blood from the lungs to the heart would have on the respira-

TABLE III.

Dog 6.

February 9, 1926.   Sudden Compression of Pulmonary Artery in an Unanesthetized Dog.

Time	Tension in cuff	Respiratory rate	Pulse rate	Remarks
a.m.	*mm. Hg*	*per min.*	*per min.*	
11.30	0	19	135	Dog quiet
11.35	0	19	168	
11.36	0	16	124	
11.39	0	18		
11.40	100±	0		Cuff abruptly inflated. Respirations ceased. Cuff released
11.41	0	20		
11.43	0	20	124	

TABLE IV.

Dog 6.

February 9, 1926.   Gradual Compression of Pulmonary Artery in an Unanesthetized Dog.

Time	Tension in cuff	Respiratory rate	Pulse rate	Remarks
p.m.	*mm. Hg*	*per min.*	*per min.*	
3.22	0	20		Dog quiet
3.23	0	22		
3.24	0			Inflation begun
3.25	40	24		
3.30	36	20	152	
3.32	50	21	147	
3.35	47	24		
3.38	62	24		
3.43	74	23		
3.45		24	145	
3.49	85	24	176	
3.52	106	26–0		Respirations ceased
3.58	0	20		

tory rhythm.   Multiple embolism of the pulmonary capillaries and arterioles, we know, results in a condition of congestion and edema of

the lungs.   That this change in structure might be responsible for the
altered function was long suspected.   Drinker, Peabody and Blum-
gart (10) showed that compression of the pulmonary veins in the cat
resulted in a diminution in lung volume as measured by the volume of
air which could enter the lungs under a uniform pressure.   This
diminution was attributed to the state of engorgement induced in the
pulmonary capillaries.   When the resistance to the outflow of blood
was sufficiently severe and prolonged a condition of pulmonary edema
arose.   Their work included no functional studies on respiration, as

TEXT-FIG. 2. Graphic spirometer tracing made by cat in Experiment 11
The first portion of tracing was taken during the control period. The second
portion of the tracing was taken toward the latter part of the experiment in
which the pulmonary veins were being progressively compressed by a ligature.
At *a* and *b* the ligature was still further tightened. The intervening respiratory
rates may be seen in Text-fig. 3.

the animals (cats) were curarized and, therefore, not breathing spon-
taneously.
   We again availed ourselves of the Drinker exposed heart prepara-
tion, and in a series of experiments on cats, we gradually compressed
the pulmonary veins and observed the effect on the heart and on the
respirations.   To our surprise exactly the same "all or none" relation-
ship obtained as in compression of the pulmonary artery.   Gradually
increased pressure exerted by a clamp and ligature on the pulmonary
veins leads, after a certain point, to acute dilatation of the heart,
followed by cessation of respiration.   In no experiment, even when
pressure was exerted for many minutes at the point just before respira-

tory failure occurs, did respirations become rapid and shallow. In Text-fig. 2 a graphic tracing is shown of the respirations and heart beat during gradual compression of the pulmonary veins in a cat. The first part of the record is the control period with the ligature in place but slack. It corresponds to the first bracketed zone in Text-fig. 3. The second portion of the record represents heart rate and respirations toward the end of compression corresponding in time to the second bracketed zone in Text-fig. 3. The intervening portion of the graphic spirometer tracing is omitted, but the rate may be seen in Text-fig. 3. During this whole second period the heart was blue and markedly dilated. Respirations will be seen to continue at about uniform rate
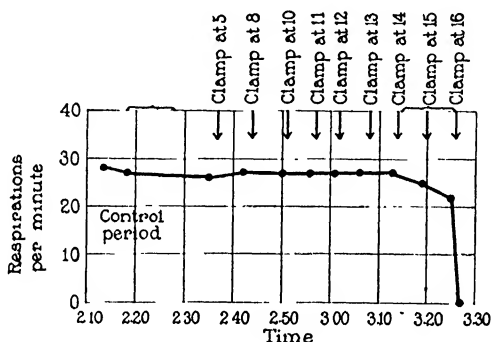
Text-Fig. 3. The respiratory rate of the cat in Experiment 11. The bracketed areas in the curve correspond in time to the two sections of the graphic tracing shown here (Text-fig. 2).

and depth until they more or less suddenly cease. This observation was repeated in two other cats in which it was shown that release of the ligature was promptly followed by return of the heart to its normal appearance and the respirations to the control rate and depth. *In no experiments did rapid and shallow breathing develop as the result of gradual compression of the pulmonary veins or, in other words, of increased resistance to the flow of blood from the lungs.* It was, unfortunately, impossible to compress the pulmonary veins in the dog because, as has been said, this animal is anatomically unsuited to the Drinker exposed heart technique; nor is the pneumatic cuff method applicable, so obscurely and closely packed are the veins as they enter the posterior auricular wall.

Since this paper constitutes the last of this series of experimental studies on respiration it may be well, even at the cost of redundancy, to consider again our findings, and what lessons are to be gleaned from them. The work was originally undertaken with a hope of understanding more clearly the mechanism and significance of that type of breathing frequently encountered in patients suffering from lobar pneumonia,—breathing characterized by being both rapid and shallow. Such disordered respiration is known to be accompanied often by oxygen want, and the point has been raised that it may indeed be responsible for oxygen want which, in turn, through its influence on the respiratory center, may perpetuate the disordered type of breathing and lead eventually to the exhaustion and ultimate collapse of the respiratory organ system.

Our work began some years ago with a quantitative study of the degree of pulmonary involvement which occurred in lobar pneumonia (11). By measuring the so called "functional residual air," or that volume of air enclosed in the lungs at the end of a normal, quiet expiration, we arrived at a more or less quantitative estimation of the degree of pulmonary involvement. It was found in general that this function varied with the clinical course of the disease, that after crisis the volume of the functional residual air rapidly increased, and that it remained either stationary or decreased as long as the active infection persisted. No unequivocal correlation, however, could be arrived at between lung volume, so measured, and the type of breathing. Occasionally rapid and shallow respirations were seen to persist for a considerable length of time after the disease process had become arrested. Nor did the state of anoxemia seem to be responsible for the accelerated respirations. In most patients in whom oxygen inhalation relieves the existing state of anoxemia, as determined by oxygen analysis of the arterial blood, rapid respirations still persist.

There seemed to be a hitherto unconsidered element responsible for the disordered breathing and for various reasons this was assumed to be one of a nervous reflex nature. The work of Porter and Newburgh (12) showed that the dyspnea associated with experimental lobar pneumonia in dogs could be checked by sectioning the vagus

nerves or blocking them with cocaine. Dunn (13) discovered the interesting fact that multiple embolism of the pulmonary arterioles and capillaries in goats resulted in very rapid and shallow breathing which could at once be stopped by double vagotomy. Our studies began where Dunn's left off. We found that this phenomenon was inherently related to obstruction of the capillaries and arterioles. When the larger branches of the pulmonary artery were obstructed a similar disorder of breathing resulted, but this was found to be wholly the result of oxygen want and could accordingly be arrested or prevented by oxygen inhalation. Anoxemia of this origin was demonstrated to be due to a relative change in rate of blood flow through the pulmonary vessels. Obstruction of the finer vessels, though often associated with anoxemia, was not in this respect the responsible factor for the disordered breathing, since rapid and shallow breathing persisted even after relief of oxygen want.

What then was the cause of rapid and shallow breathing due to capillary embolism? To this problem we addressed ourselves and it was found far more difficult in its solution than had been anticipated. Indeed a proper understanding of it has depended upon the exhibition of a large amount of so called negative evidence.

We know that capillary obstruction is associated with a diminution in the volume of the "functional residual air" which is probably dependent upon the secondary state of edema, atelectasis and congestion which arises. It seemed reasonable to believe that this pathological condition of the lung, which could give rise to oxygen want, might likewise be responsible for a retention of carbon dioxide, or an increase in the pressure of CO_2 and the concentration of hydrogen ions in the blood. These changes were, in fact, found but that they are not the cause of the disordered breathing is strongly indicated by the fact that rapid and shallow breathing may occur after embolism without any increase in pCO_2 or fall in pH. We know, too, that the actual presence of the embolic material is not responsible for the accelerated breathing in the sense of its being a local irritant, though the inhibitory effect of vagotomy or vagal freezing first made us suspect this to be true. The fact that starch emboli do not produce rapid and shallow breathing solely by acting as local irritants is shown by these two considerations: (1) a considerable amount of embolic mate-

rial may be present without any influence upon the normal respiratory rhythm; (2) emboli may be present in the capillaries and arterioles of one lung sufficient ordinarily to produce rapid breathing without however, any alteration in normal breathing unless the circulation to the other lung is cut off or has been disturbed by a previous period of obstruction. These last facts suggested that the phenomenon was in some manner related to (1) a restriction of the pulmonary vascular bed, (2) resistance to the flow of blood through the lungs or (3) the state of congestion and edema of the lungs. The first two of these possibilities might easily be related to an extra burden on the heart, resulting in an increased pressure in the pulmonary artery and right side of the heart with a subsequent dilatation of these tissues. The demonstration by Levy (7) of an increased heart size in pneumonia was thought perhaps to be a germane phenomenon. It has been shown in this paper that dilatation of the pulmonary artery and right heart does in fact occur when the arterioles and capillaries of the lungs are obstructed. And it was tentatively hypothecated that the dilatation of the pulmonary artery and right heart resulting from obstruction to the finer vessels of the lungs occasioned impulses which reflexly accelerated respirations. Evidence has been brought forth in this paper to show that this hypothesis is erroneous and that reduction of the pulmonary vascular bed, at least by half, produces no change in breathing. Moreover, both resistance to the flow of blood to and from the lungs though followed, when of sufficient grade, by dilatation of the pulmonary artery and right heart, does not give rise to rapid and shallow breathing. The result of such resistance is an "all or none" phenomenon in which respirations continue at their normal rate and depth until they more or less suddenly cease completely.

We are left then to a consideration of the third possibility enumerated above, namely, that rapid and shallow breathing of the kind here described is the result of the particular lesion produced in the lungs, namely, congestion and edema.

How this acts to produce accelerated breathing still remains more or less a mystery: whether directly through irritation of vagal nerve ending; whether as the result of marked encroachment on lung volume; or whether through changes in elasticity of the lung, which to be sure we have not been able to demonstrate in the embolized lung at autopsy.

The first of these three possibilities seems to us at present to be the most likely. It is tempting to theorize but perhaps unwise to befog an already complicated subject by unproven hypotheses. Certainly congestion of the lungs, reduction in lung volume and impaired elasticity of the pulmonary parenchyma are all three intimately associated phenomena, and are present in many of the clinical states in which disordered breathing is a prominent symptom.

SUMMARY AND CONCLUSIONS.

1. Embolism of pulmonary arterioles and capillaries produced by the intravenous injection of starch grains results in a dilatation of the pulmonary artery and the right chambers of the heart. This has been demonstrated both by x-ray studies and direct inspection.

2. The dilatation of the pulmonary artery and heart occurs synchronously with the acceleration of respirations.

3. Dilatation of these structures produced by other means, such as obstruction to the flow of blood to and from the lungs, by gradually clamping either the pulmonary artery (cat and dog) or pulmonary veins (cat) does not, however, give rise to rapid and shallow breathing.

4. The effect of these maneuvers on respiration does not become apparent until respirations suddenly cease.

5. Neither does sudden restriction of the pulmonary vascular bed by clamping the left branch of the pulmonary artery give rise to rapid and shallow breathing, though this procedure may cause an increase in CO_2 tension and in hydrogen ion concentration of the blood.

6. Since rapid and shallow breathing is *not* the result of (1) anoxemia, (2) increased pCO_2 and hydrogen ion concentration of the serum, (3) restriction of pulmonary vascular bed by nearly half, (4) increase in resistance to the flow of blood to and from the lungs, (5) the presence of starch grains in the lungs acting as a local irritant, it must be the result of the secondary pathological changes which occur in the pulmonary parenchyma following embolism.

7. The nature of these changes, congestion and edema, has been discussed elsewhere. Whether they operate directly on nerve endings or through their influence on lung volume and tissue elasticity is not certain.

8. Various important clinical analogies have been emphasized.

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EXPLANATION OF PLATES.

PLATE 24.

FIG. 1, *a* and *b*. X-ray photograph of dog's heart taken in Experiment 1. Tube distance 2 meters. In *a* the dog was breathing 16 times to the minute, the area of the shadow as measured by a planimeter being 43.4 sq. cm. In *b* after a starch suspension had been injected intravenously, respirations were 64 to the minute, and the area of the shadow is 48.4 sq. cm.

PLATE 25.

FIG. 2. Operative field with pulmonary artery exposed and cuff in place ready for ligation.

FIG. 3. Dog 6, 8 days after operation, showing tube, which communicates with cuff surrounding the pulmonary artery, projecting through the chest wall.

Fig. 1, b.

Fig. 1, a.

(Moore and Binger: Blood flow to and from lungs.)
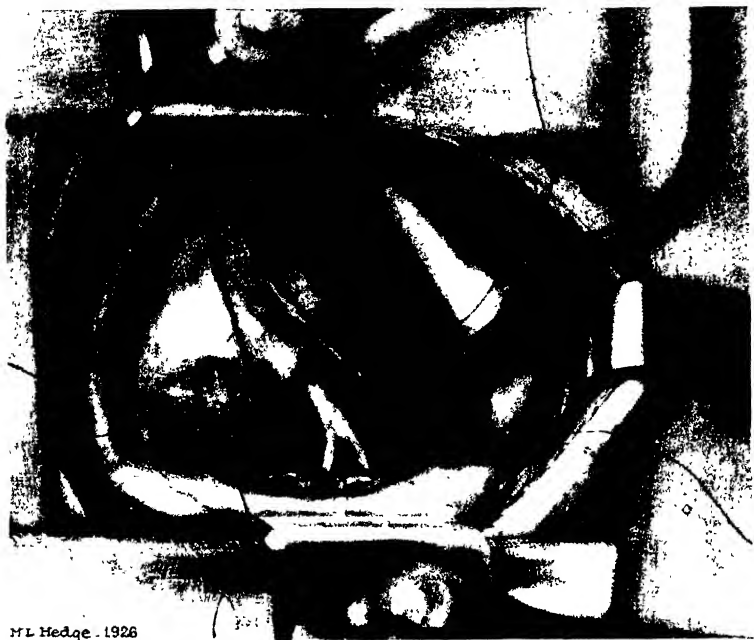
FIG. 2.
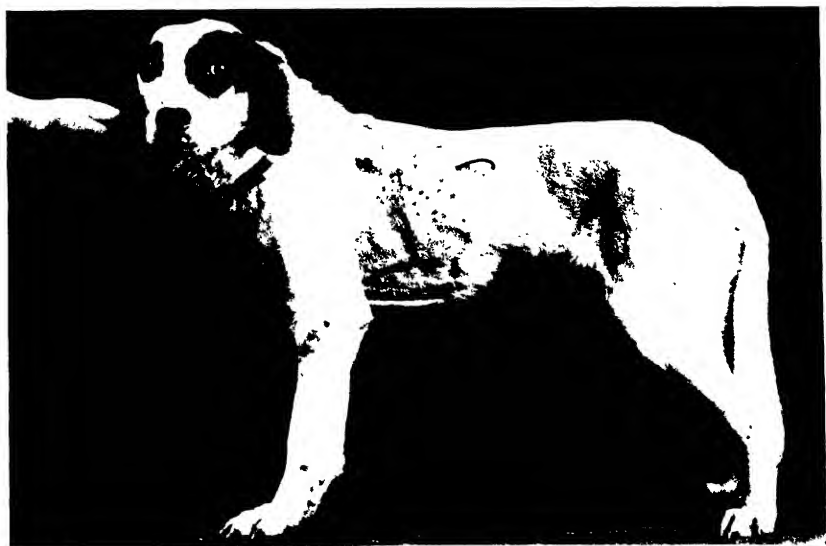
FIG. 3.

(Moore and Binger: Blood flow to and from lungs.)

STUDIES ON IMMUNITY TO PNEUMOCOCCUS MUCOSUS (TYPE III).

I. ANTIBODY RESPONSE OF RABBITS IMMUNIZED WITH TYPE III PNEUMOCOCCUS.

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The absence of demonstrable type specific antibodies in the serum of rabbits immunized with Type III pneumococcus has been the common experience of investigators working with this organism (1–4). Yoshioka (5) obtained type specific agglutinins in the serum of immunized rabbits, but with irregularity. He attributed his positive results to the use of one strain of Type III, since, with two other strains, no specific antibody production was elicited. Immunization of sheep and horses with Type III pneumococcus has resulted in the development of type specific agglutinins in the sera of these animals (6), but the titre has been low and passive protection slight.

This experience with *Pneumococcus mucosus* is but a confirmation of the earlier observations (7) that the serum of animals immunized with certain of the encapsulated organisms fails to show agglutinins. This failure to demonstrate antibodies in serum under these conditions has been usually attributed to the inagglutinability of organisms possessing a mucoid capsule or to the absence of agglutinins in the test serum. Porges (8) found that if encapsulated Friedländer bacilli were first subjected to a process of decapsulation, they then became agglutinable in immune serum. Hanes (1) reported that Type III pneumococci were agglutinated in their homologous immune serum following decapsulation of the organisms by means of the Porges method.

In the course of a series of investigations on immunity to Type III pneumococcus, the response of rabbits to immunization with this organism was investigated.

EXPERIMENTAL.

Antigen.—Three different strains of Type III pneumococcus were employed. These strains were derived from the blood of patients suffering from lobar pneumonia and possessed all the characteristics of Type III. They were Gram-positive diplococci; they showed capsule formation when suitably stained; they grew on blood agar plates with the production of large mucoid colonies; they were bile-soluble; they agglutinated promptly in Type III antipneumococcus horse serum; they were virulent for mice in 0.0000001 cc. doses. Both living organisms and vaccine were used for injection. A 16 hour plain broth culture was used for the living organisms. The vaccine was made by centrifuging such a culture, resuspending the organisms in physiological salt solution, and heating at 56° for half an hour.

Methods of Immunization.—Three methods of immunization were employed. (1) The intravenous injection of vaccine according to the method described by Cole and Moore (9). This method consists in alternating for 6 weeks a week of daily injections of 1 cc. of vaccine with a week of rest. (2) Following a week of intravenous vaccine injections of 1 cc. each day, living organisms were injected at 3 to 4 day intervals in increasing amounts from 0.1 to 10 cc. over a period of 4 weeks. (3) Living organisms were injected intravenously from the beginning of immunization; 1 cc. was given daily for 6 days followed by increasing doses from 3 to 10 cc. at 3 to 4 day intervals over a period of 4 weeks.

All animals were bled 10 to 12 days after the last immunizing dose.

Agglutinin Reaction.—0.5 cc. of a saline suspension of heat-killed organisms was added to an equal volume of the serum dilutions. In the tests with type specific pneumococci, a positive reaction, if present, usually appeared immediately and the final reading was made after the tubes had remained 2 hours in the water bath at 37°C. When decapsulated Type III pneumococci or R strains of pneumococci were used, the reading was made after the tubes had remained 2 hours in the water bath at 37°C. and overnight in the ice box.

Precipitin Reaction. Nucleoprotein.—The nucleoprotein solutions of Pnuemococcus used as precipitinogen in the tests were made by the method described by Avery and Morgan (10). They contained approximately 3 mg. of N per cc. 0.2 cc. of the test serum diluted to 0.5 cc. by the addition of physiological salt solution was added to 0.5 cc. of the nucleoprotein solution. Readings were made after 2 hours in the water bath at 37°C. and overnight in the ice box.

Soluble Specific Substance.—This substance, representing the N-free carbohydrate derived in a purified state from Type III pneumococci according to the method described by Heidelberger and Avery (11), was used in dilutions of 1–10,000, 1–20,000, 1–40,000, and 1–100,000. Dilutions of this degree were employed in order to escape the inhibition zone experienced (12) when higher concentrations are tested. 0.2 cc. of serum diluted to 0.5 cc. by the addition of physiological salt solution was added to 0.5 cc. of the different dilutions of the soluble specific substance. The precipitin reaction is usually immediate; however, the final reading was made after 2 hours in the water bath at 37°C.

Passive Protection Tests.—These tests were carried out in mice by the simultaneous injection intraperitoneally of 0.2 cc. of serum and culture dilution, according to the method previously described (13).

The results obtained in the experiments to be reported involve the activities of two antigen-antibody systems. In order to avoid confusion as to nomenclature, certain terms used throughout the body of the report will be defined. It has been shown by Avery and Heidelberger (14), that pneumococci are capable of stimulating the production of two distinct antibodies, depending in a large measure on the nature of the bacterial substances used as antigens. An intact pneumococcal cell possessing the soluble specific substance, when employed antigenically, stimulates the production of antibodies which are reactive with the homologous soluble specific substance. If the soluble specific substance is present in the cells used in the agglutination test, type specific agglutination occurs; if the soluble specific substance is present in solution, precipitation of this substance occurs. The antibody, which is capable of agglutinating the type specific Pneumococcus and of precipitating the type specific soluble substance will be designated as *anti-S*. The evidence of all previous work indicates that the efficacy of serum in the passive protection of mice against pneumococcus infection depends on the presence in the serum of anti-S.

The second antigen-antibody system involves the nucleoprotein fraction of the cell and the antibody response elicited by it. Pneumococcus nucleoprotein, in contrast to the soluble specific substances, is without type specificity. The degraded R strains of pneumococci also are without type specificity. Therefore the use, antigenically, of either nucleoprotein substance or R strains results in the production of non-type specific antibodies, which precipitate the nucleoprotein derived from any Pneumococcus and agglutinate all R strains. These antibodies will be designated as *anti-P*.

I. Agglutinins.

Absence of Agglutinins for the Encapsulated Type III Pneumococcus in the Sera of Animals Immunized with Type III Pneumococcus.

Twenty-eight rabbits were used in these immunization experiments. Twenty received vaccine alone; six received vaccine and living organ-

isms; two received living organisms alone. Their sera were then tested for the presence of agglutinins against the encapsulated Type III pneumococcus. Out of the twenty-eight rabbits, the sera of twenty-four failed to show type specific antibodies (anti-S). In the four positive sera type specific antibodies (anti-S) were present in low titre and in only one was the agglutinin reaction positive in a dilution of 1 to 20 (Table I). Such results are confirmatory of the experience of others in working with Type III and are in striking contrast to the results obtained when rabbits are immunized with Pneumococcus Type I or Type II, whereby type specific antibodies are readily demonstrable in the immune sera.

Presence of Agglutinins for R Strains of Pneumococcus in the Sera of Rabbits Immunized with Type III Pneumococcus.

In demonstrating the twofold antigenicity of pneumococcus substances, Avery and Heidelberger (14) showed that when intact pneumococcal cells are used as antigen the resultant antibodies consist chiefly of type specific agglutinins (anti-S) for the homologous organism; when ruptured cells or solutions of pneumococci are used as antigen the antibody response is predominantly of the anti-P character. Since the sera of rabbits immunized with Type III pneumococcus fail, in most instances, to show anti-S antibodies, and in view of the dual antigenic nature of pneumococci, these immune sera were tested for the presence of anti-P antibodies. Anti-P antibodies are reactive against the degraded R strains of Pneumococcus. As has been brought out by Reimann (15, 16) R strains are avirulent, non-encapsulated, and non-type specific. They may be derived from any of the fixed types of Pneumococcus and are agglutinated by any anti-P serum. Therefore, R strains of pneumococci are adequate in testing for the presence of anti-P antibodies. The results recorded in Table I were obtained by testing the sera of rabbits, immunized with Type III pneumococcus, for the presence of anti-P antibodies, as evidenced by the agglutination of an R strain. The sera of twenty-seven of twenty-eight rabbits possessed anti-P antibodies.

The results of this experiment indicate that rabbits immunized with Type III pneumococcus—even though this organism is one of the fixed types—react in the majority of instances by the production, not

of the type specific anti-S antibody, but of the anti-P antibody. The immunological response is identical with that obtained following immunization with R strains or solutions of pneumococci. The inference is, then, that Type III pneumococci are so altered, following introduction into the animal body, that the type specific antigen is made ineffectual and the nucleoprotein is liberated to stimulate the production of anti-P antibodies.

Agglutination of Decapsulated Pneumococci in the Sera of Rabbits Immunized with: (1) Type III Pneumococcus, (2) R Strains of Pneumococcus, (3) Nucleoprotein Derived from Pneumococcus.

Hanes (1), as previously stated, showed that Type III pneumococci, deprived of their capsule by means of the Porges (8) method, were agglutinated in the sera of rabbits immunized with Type III pneumococci; whereas, when the encapsulated organisms were tested in the same immune sera, no agglutination occurred. Both Porges and Hanes infer that the inagglutinability of encapsulated organisms is due, not to the absence of agglutinins in the test serum, but to the presence of the capsule around the organism. As proof of this they show that removal of the capsule is followed by agglutination.

Since it has been shown in Table I that rabbits immunized with Type III pneumococcus possess anti-P antibodies and, since the chief characteristic of anti-P antibodies is the agglutination of non-encapsulated.pneumococci, an explanation of the results of Hanes is made possible. R strains of pneumococci agglutinated by Type III immune rabbit serum (Table I) are organisms which have been deprived of their capsule by cultural methods. The Type III organisms which Hanes found agglutinable were deprived of their capsule by a chemical procedure. The agglutination of both of these non-encapsulated organisms is identical (Table I) in Type III immune rabbit serum. Their immunological identity is further brought out by testing the agglutination of chemically decapsulated organisms in the sera of rabbits immunized with R strains and with pneumococcus nucleoprotein. The results (Table II) show that any anti-P serum is capable of agglutinating the chemically decapsulated Type III pneumococcus and R strains equally well. In reports on Friedländer bacilli Julianelle (17) obtained similar results; namely, that decapsulated and R

TABLE I

Agglutination of Encapsulated Type III Pneumococcus, Decapsulated Type III Pneumococcus, and of an R Strain of Pneumococcus in the Sera of Rabbits Immunized with Type III Pneumococcus.

Rabbit No.	Agglutinins for Pneumococcus III encapsulated — Serum dilutions					Agglutinins for Pneumococcus III decapsulated — Serum dilutions					Agglutinins for R₃ strain of pneumococcus — Serum dilutions					
	1-2	1-10	1-20	1-40	1-80	1-10	1-20	1-40	1-80	1-160	1-2	1-10	1-20	1-40	1-80	1-160
1	−	−	−	−	−	+++	+++	+	±	−	+++	+++	++	++	±	−
2	−	−	−	−	−	+++	+++	++	+	+	+++	+++	++	++	−	−
3	−	−	−	−	−	+++	++	+	−	−	+++	+++	++	++	−	−
4	−	−	−	−	−	+++	+++	+++	±	−	+++	+++	++	++	+	−
5	−	−	−	−	−	+++	+++	+++	±	±	+++	+++	++	±	−	±
6	−	−	−	−	−	+++	+++	+	±	−	+++	+++	++	−	−	−
7	−	−	−	−	−						+++	+++	++	++	+	−
8	−	−	−	−	−						+++	++	±	++	−	−
9	−	−	−	−	−						+++	+++	++	++	±	−
10	−	−	−	−	−						+++	+++	++	++	−	−
11	−	−	−	−	−						+++	++	++	++	±	−
12	−	−	−	−	−						+++	+++	+++	++	−	−
13	−	−	−	−	−						+++	+++	+++	++	+	−
14	−	−	−	×	−						+++	+++	+++	++	−	−
15	××××	××	×	×	−						−	−	−	−	−	−
16	×××	×	−	−	−						+++	++	+++	++	±	−
17	××	×|×|	−	−	−						+++	++	+++	+	−	−
18	−	−	−	−	−						+++	+++	+++	+	−	−
19	−	−	−	−	−						++++	−	−	−	−	−
20	−	−	−	−	−						±	+	±	±	−	−
21	−	−	−	−	−						++++	++	++	−	±	−
22	−	−	−	−	−						++++	+++	+	±	−	−

23	—	—	—	—	+++	+++	++	++	—
24	—	—	—	—	+++	+++	+++	++	+	±
25	—	—	—	—	+++	+++	++	++	—
26	—	—	—	—	+++	++	+	±	±
27	××××	××	×	—	+++	++	++	+	—
28	—	—	—	—	+++	+++	++	±	±

Controls: 0.5 cc. organisms + 0.5 cc. of salt solution: diffuse. 0.5 cc. organisms + 0.5 cc. of normal rabbit serum: negative.

×××× indicates characteristic compact disc of type specific agglutination with clear supernatant fluid. ××× indicates compact disc with faintly cloudy supernatant fluid. ×× indicates disc which can be partially broken up with cloudy supernatant fluid. × indicates coarse granulation. $\underline{\times}$ indicates doubtful granulation. — indicates negative. ++++ indicates complete agglutination with clear supernatant fluid. +++ indicates less coarse agglutination with faintly cloudy supernatant fluid. ++ indicates agglutination with cloudy supernatant fluid. + indicates some granulation both sedimented and diffuse. ± indicates some sedimentation easily made diffuse. — indicates negative.

TABLE II.

Agglutination of Decapsulated Type III Pneumococci and of R Pneumococci in the Sera of Rabbits Immunized with Two Different R Strains of Pneumococcus and with Pneumococcus Nucleoprotein.

Rabbit No.	Immunized with	Decapsulated Type III						R₂ pneumococci					
		1-10	1-20	1-40	1-80	1-160	1-320	1-10	1-20	1-40	1-80	1-160	1-320
29	R_1 strain	++++	++++	+++	+	±	-	++++	+++	++	+	±	-
30	R_2 strain	++++	+++	+	±	-	-	++++	+++	+	-	-	-
31	Nucleo-protein	++++	+++	++	+	±	-	++++	+++	++	+	-	-

Controls: 0.5 cc. of organisms + 0.5 cc. of salt solution: diffuse. 0.5 cc. of organisms + 0.5 cc. of normal rabbit serum: some sedimentation which was made diffuse by slight tapping.

++++ indicates complete agglutination with clear supernatant fluid. +++ indicates less coarse agglutination with faintly cloudy supernatant fluid. ++ indicates agglutination with cloudy supernatant fluid. + indicates some granulation both sedimented and diffuse. ± indicates some sedimentation easily made diffuse. − indicates negative.

strains of Friedländer bacilli were identical in their agglutination reactivity in anti-P (Friedländer) serum.

II. Precipitins.

It was shown by Reimann (16) that sera which contained agglutinins (anti-P) for R strains of Pneumococcus also contained precipitins for pneumococcus nucleoprotein solutions. Therefore, since the sera of the rabbits immunized with Type III pneumococcus possessed anti-P agglutinins, they were tested for the presence of precipitins for pneumococcus nucleoprotein (Table III).

A comparison of Table III with Table I will show that all the sera which contained anti-P agglutinins also possessed anti-P precipitins. Since the nucleoprotein in this test was obtained from a Group IV pneumococcus, the possibility of a type specific reaction is excluded and the experiment further identifies the antibody present in the Type III immune sera as of an antiprotein character.

The sera of the rabbits immunized to Type III were also tested for the presence of precipitins against Type III specific soluble substance (Table III). As has been shown by Avery and Heidelberger (14), the type specificity of pneumococci depends upon the presence of this substance. They have obtained it in a highly purified state from each of the three fixed types of Pneumococcus. Immune sera which possess type specific agglutinins (anti-S) for pneumococci of a fixed type also possess precipitins for the soluble specific substance derived from that type. The results obtained with Type III immune rabbit sera used in these experiments show the concomitant occurrence of Type III specific agglutinins (anti-S) and precipitins for the Type III soluble specific substance. On the other hand, those sera which failed to show type specific agglutinins (anti-S) also failed to precipitate the soluble specific substance. This is further evidence that the lack of agglutination of the encapsulated Type III cell in homologous immune rabbit serum is due to the actual absence of the anti-S antibody in demonstrable quantity since such factors as were supposed to render the encapsulated organism inagglutinable are not present in the precipitin test.

TABLE III.

Precipitation of the Soluble Specific Substance of Type III Pneumococcus and of a Solution of Pneumococcus Nucleoprotein in the Sera of Rabbits Immunized with Type III Pneumococcus.

Rabbit No.	Precipitins for soluble specific substance of Type III pneumococcus				Precipitins for solution of pneumococcus nucleoprotein
	Dilutions of soluble specific substance				Dilution of protein
	1–10,000	1–20,000	1–40,000	1–100,000	1–300
1	−	−	−	−	+++
2	−	−	−	−	++
3	−	−	−	−	++
4	−	−	−	−	++
5	−	−	−	−	+++
6	−	−	−	−	++
7	−	−	−	−	++
8	−	−	−	−	+++
9	−	−	−	−	++
10	−	−	−	−	++
11	−	−	−	−	++
12	−	−	−	−	++
13	−	−	−	−	++
14	−	−	−	−	++
15	××××	××	×	$\underline{\times}$	+
16	××××	×	×	−	±
17	×××	×	−	−	++
18	−	−	−	−	++
19	−	−	−	−	++
20	−	−	−	−	−
21	−	−	−	−	+
22	−	−	−	−	++
23	−	−	−	−	++
24	−	−	−	−	+++
25	−	−	−	−	++
26	−	−	−	−	++
27	××××	×	×	−	++
28	−	−	−	−	+++

Controls: 0.5 cc. of various dilutions of soluble specific substance + 0.5 cc. salt solution: clear. 0.5 cc. of various dilutions of soluble specific substance +0.2 cc. of normal rabbit serum + 0.3 cc. of salt solution: clear. 0.5 cc. of pneumococcus nucleoprotein solution + 0.5 cc. of salt solution: slightly hazy.

III. Passive Protection in Mice.

The sera of the twenty-four rabbits immunized with Type III pneumococcus were tested for their ability to protect mice against infection with Type III pneumococcus. The dependence of passive protection on the presence of type specific antibodies (anti-S) in the test serum is well known to investigators working in experimental pneumococcus immunity. Therefore, it is to be expected that those sera containing anti-S afford some protection and that those without it fail. The results recorded in Table IV show that protection parallels the presence of anti-S and not anti-P. Of the twenty-four sera tested, the four possessing anti-S showed protection; of the twenty possessing no demonstrable anti-S, seventeen afforded no protection, and three afforded very slight protection. The explanation of the results obtained with the latter three sera probably rests on the fact that these sera contained sufficient type specific antibodies (anti-S) to afford some protection but not sufficient to be demonstrable by test-tube agglutination. That the mouse protection test is a more delicate test for the presence of anti-S than test-tube agglutination may be brought out by the use of Type I antipneumococcus serum. This serum may be diluted to a point where no test-tube agglutination is demonstrable and yet such a dilution of serum affords some protection. A further report will be made later on the results obtained in passive protection following the use of the sera of rabbits immunized with Type III pneumococci.

An analysis of the antibodies present in the sera of twenty-eight

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0.5 cc. of pneumococcus nucleoprotein solution + 0.2 cc. of normal rabbit serum + 0.3 cc. of salt solution: slightly hazy.

X X X X indicates characteristic compact disc of type specific precipitin reaction with clear supernatant fluid. X X X indicates compact disc with faintly cloudy supernatant fluid. X X indicates smaller disc at bottom of tube with cloudy supernatant fluid. X indicates cloudy fluid. X indicates very faintly cloudy fluid. — indicates negative.

+ + + indicates precipitate at bottom of tube with faintly cloudy supernatant fluid. + + indicates slight precipitate with cloudy supernatant fluid. + indicates cloudy fluid. ± indicates very faintly cloudy fluid. — indicates negative.

rabbits immunized with Type III pneumococcus may be summarized as follows:

Agglutinins.—Twenty-four failed to show type specific agglutinins (anti-S). Four possessed type specific agglutinins (anti-S) in low titre. Twenty-seven possessed anti-P agglutinins in appreciable titre. One failed to show evidence of any antibody response.

TABLE IV.

Summary of Passive Protection in Mice by the Use of Sera of Rabbits Immunized with Type III Pneumococcus.

	Demonstrable anti-S antibodies		Demonstrable anti-P antibodies		Rabbit No.	Passive protection in mice against Type III pneumococcus infection			
						Dose of culture			
	Agglutinins	Precipitins	Agglutinins	Precipitins		0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.
Sera of 17 rabbits	Absent	Absent	Present	Present	.	D.	D.	D.	D.
Sera of 3 rabbits	Absent	Absent	Present	Present	6 11 12	D. D. D.	D. D. D.	D. D. S.	S. S. S.
Sera of 4 rabbits	Present	Present	Present	Present	15 16 17 27	S. S. D. D.	S. S. S. S.	S. S. S. S.	S. S. S. S.

D. indicates death of animal.
S. indicates survival of animal.

Precipitins.—The presence of precipitins for Type III soluble specific substance paralleled the presence of type specific agglutinins. The presence of precipitins for pneumococcus nucleoprotein paralleled the presence of anti-P agglutinins.

Passive Protection in Mice.—Four sera possessing demonstrable anti-S antibodies afforded some protection against Type III infection.

Of twenty sera without demonstrable anti-S, seventeen failed to confer any protection; three afforded minimal protection.

DISCUSSION.

The common experience that Type III pneumococci, antigenically employed, fail to stimulate the production of type specific agglutinins (anti-S) has been encountered in a great majority (85.8 per cent) of the rabbits used in the experiments here reported.   It is a striking fact that such a cell, possessing a large amount of soluble specific substance, and highly virulent for mice, acts so feebly in stimulating type specific antibodies (anti-S); whereas Type I and Type II pneumococci, possessing the same qualities, are so effective in producing homologous type specific antibodies.   However, the presence of agglutinins for R pneumococci and precipitins for pneumococcus nucleoprotein (anti-P antibodies) is evidence that Type III pneumococci are not without an antigenic component.   The results of these experiments reveal the fact that the antibody response of rabbits to immunization with the encapsulated Type III pneumococcus is identical with the antibody response obtained by immunization with solutions of pneumococci.   In both instances the production of anti-P antibodies has been stimulated.   Such a result could be accomplished only by *in vivo* disruption of the pneumococcal cells.   The inference, then, may be drawn that normal rabbits possess a mechanism whereby Type III pneumococci, following intravenous injection, are disintegrated in such a manner that the part of Type III antigenic complex, which stimulates type specific agglutinins (anti-S), is destroyed.   The nucleoprotein fraction of the cell, however, remains capable of stimulating anti-P antibodies.

The identification of the antibody present in Type III immune rabbit serum, as being anti-P, is explanatory of Hanes' results with decapsulated Type III pneumococci.   By decapsulation, Type III pneumococci are reduced to an R form, and are agglutinated by anti-P antibodies.   The failure of encapsulated Type III to agglutinate is due to the actual absence of anti-S antibodies.

CONCLUSIONS.

1. Type III pneumococci fail in the majority of instances to stimulate the production of anti-S antibodies.   (Type specific agglutinins, type specific precipitins, and antibodies affording type specific protection in mice.)

2. Type III pneumococci are effective in the stimulation of the production of anti-P antibodies (agglutinins for R strains of pneumococci and precipitins for pneumococcus nucleoprotein). These antibodies are ineffectual in the passive protection of mice.

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ON THE OXIDATION OF GLUCOSE IN ALKALINE SOLUTIONS OF IODINE.

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In 1918 Willstätter and Schudel (1) described a method for the estimation of sugars which embodied the principle first set forth by Romijn (2), that hexo-aldoses may be quantitatively oxidized to the corresponding hexonic acid in the presence of iodine in alkaline solution. The reaction may be expressed by the equation $RCHO + I_2 + 3NaOH = RCOONa + 2NaI + 2H_2O$. Thus 1 molecule of aldose reacts with 2 molecules of sodium hypoiodite, and when a measured excess of the latter is used the amount of iodine liberated on acidification, after the reaction between aldose and hypoiodite has reached completion, serves as a measure of the quantity of sugar present.

In the original description of their method Willstätter and Schudel state that sodium hydroxide must be slowly added to the sugar-iodine solution. It was observed by the author that if sodium hydroxide be added immediately to the glucose-iodine solution, or even over an interval of 30 to 60 seconds, one does not obtain quantitative oxidation of glucose. The success of the method depends therefore primarily on the rate of addition of sodium hydroxide to the mixture of glucose-iodine. These observations are summarized in Table I.

It is clearly seen that the rate of addition of alkali has a distinct influence on the success of the reaction glucose→gluconic acid in reaching completion. If solutions of glucose-iodine, to which alkali has immediately been added, as in Experiments 6 to 8, are permitted to stand longer than 15 minutes, the oxidation of the sugar fails to come to completion, and at the end of a prolonged period of time, an hour or more, no hypoiodite can be demonstrated in the

reaction mixture. Apparently the oxidizing reaction has reached completion with some 4 per cent of glucose still unchanged. In the preparative method found in the following paper, this effect is shown to be greatly magnified. It is the nature of this phenomenon which will be discussed.

TABLE I.

Effect of Rate of Addition of 2 Equivalents of 0.1 N Sodium Hydroxide on Oxidation of 10 Cc. of 0.9 Per Cent Glucose and 20 Cc. of 0.1 N Iodine-Potassium Iodide Solutions at 23°.

Experiment No.	Glucose.	Time over which 2 equivalents of 0.1 N NaOH were added.	0.1 N iodine utilized in oxidation of glucose.	Glucose oxidized as calculated from iodine consumption.
	mg.	*sec.*	*cc.*	*per cent*
1	90	240	10.00	100
2	90	120	10.00	100
3	90	60	9.94	99.4
4	90	30	9.87	98.7
5	90	15	9.78	97.8
6	90	Immediately.	9.65	96.5
7	90	"	9.64	96.4
8	90	"	9.65	96.5

The method of analysis in Table I was carried out as follows: To 10 cc. of a 0.9 per cent glucose solution were added 20 cc. of 0.1 N iodide-potassium iodide solution and to this were added 2 equivalents of 0.1 N sodium hydroxide (40 cc. + 5 cc. to neutralize the gluconic acid formed). When the alkali was added over an interval, it was run from a burette at constant rate. When added "immediately" the required amount was quickly poured from a cylinder into the well stirred glucose-iodine solution. The whole operation required about 3 seconds. After standing a total of 15 minutes, when the reaction had come to completion, the mixture was acidified with 6 cc. of 1.0 N hydrochloric acid and was titrated with 0.05 N sodium thiosulfate, using starch as an indicator. The method is accurate to about ±0.15 per cent. The temperature variation was not greater than ±0.25°. The sodium hydroxide used in these and subsequent experiments was exactly 0.1 N and was prepared from carbonate-free sodium hydroxide from sodium. The iodine-potassium iodide solution contained 25.0 gm. of the potassium iodide per liter.

Until the appearance of Schönbein's (3) "Beiträge zur nähern Kenntniss des Sauerstoffs und der einfachen Salzbildner" it was believed that iodine and potassium hydroxide in solution reacted

instantaneously to form potassium iodate. Schönbein showed this reaction to take place in two stages, and from the analogy between the chemical similarity of iodine and chlorine, assumed that an intermediate product, potassium hypoiodite, was formed. Schönbein's experiments showed that the first stage of the reaction, the formation of hypoiodite, is incomplete and reversible, and that an equilibrium between alkali, iodine, iodide, and hypoiodite existed. With regard to the second reaction, the formation of iodate from hypoiodite, he says little except that the velocity is increased as the temperature is raised. This second reaction has been studied in great detail by many investigators and an excellent review of the literature is to be found in the articles of Skrabal (4) which appeared in 1907. Most of these studies have been carried out in solutions of high dilution, but Taylor (5) has demonstrated that in solutions of decinormal strength the formation of iodate from hypoiodite progresses at a remarkable rate.

In an attempt to obtain a more accurate knowledge of the rate of formation of iodate from hypoiodite in solutions of the same concentration as those employed in the Willstätter-Schudel method, a short series of experiments was carried out and the results are given in Table II. A second series of experiments was also carried out concerning the effect of the rate of addition of alkali on the rate of oxidation of glucose and on the rate of the formation of iodate from hypoiodite in these sugar-containing solutions. These results are found in Table III.

From the data presented in these tables there are two facts worthy of note: first, in the absence of glucose the rate of addition of 0.1 N sodium hydroxide to 0.1 N iodine solution has no influence on the speed of formation of iodate from hypoiodite over the time intervals studied; and second, the rate of addition of alkali has a marked influence on the speed of formation of iodate from hypoiodite in a solution containing glucose. It is to be borne in mind that in an alkaline glucose-iodine solution in addition to the reaction (1) $RCHO + 3NaOH + I_2 = RCOONa + 2NaI + 2H_2O$ there is going on simultaneously a second and equally important reaction (2) $3NaOI = NaIO_3 + 2NaI$. In order to explain the phenomenon of the failure of glucose to be oxidized quantitatively when alkali is im-

mediately added to glucose-iodine solution, it becomes necessary to take into consideration the reaction velocity of this second reaction. Before going on with this discussion, however, an important factor

TABLE II.

Rate of Formation of Iodate from Hypoiodite in Solution of 20 Cc. of 0.1 N Iodine, 10 Cc. of Water, and 40 Cc. of Sodium Hydroxide at 23°.

Series No.	Time of reaction.	Hypoiodite-iodide in solution.	Iodate-iodide in solution.
	min.	*per cent*	*per cent*
I	2	17.8	82.0
	7	5.8	94.2
	12	3.7	96.1
II	2	18.0	82.0
	7	4.5	95.6
	12	3.7	96.2

The method of procedure in Table II was as follows: In Series I 2 equivalents (40 cc.) of 0.1 N sodium hydroxide were immediately added to 20 cc. of 0.1 N iodine-potassium iodide solution and 10 cc. of water. The mixture was permitted to stand the required length of time, then to it were added 2 gm. of solid sodium bicarbonate and 100 cc. of water. A blast of carbon dioxide was passed through to neutralize the free alkali. In this manner the iodine from the hypoiodite and unaltered iodine was liberated. 15 cc. of 0.1 N sodium arsenite were added and the excess arsenite was titrated with 0.1 N iodine-potassium iodide solution, using starch as indicator. The back titration with 0.1 N iodine in cc. minus 15 cc. is equal to the cc. of iodine in the form of hypoiodite and iodine in equilibrium. The iodate was then determined by adding small portions of HCl to this solution and titrating with 0.05 N sodium thiosulfate. In Series II 2 equivalents of 0.1 N alkali were added at constant rate from a burette over an interval of 2 minutes, and the analyses made exactly as above. The time of reaction was counted from the first addition of sodium hydroxide. The temperature variation, as in all these experiments was not greater than ±0.25°. Since iodine in sodium hydroxide exists in equilibrium form, $2I + NaOH \rightleftharpoons NaOI + HI$, and $3NaOI = NaIO_3 + 2NaI$, the so called "hypoiodite" determination represents all of the iodine in the first equation; the "iodate" determination represents the right hand member of the second equation.

which determines the success of reaction (1) must be pointed out. This factor is the necessity for the presence, as the reaction approaches completion, of a concentration of hypoiodite sufficient to drive it to

the end.   If the concentration falls below a certain optimum, reaction (1) will never reach completion.   The condition which determines this factor is the rate of addition of sodium hydroxide.

TABLE III.

Influence of Rate of Addition of 2 Equivalents of 0.1 N Sodium Hydroxide on Rate of Formation of Iodate from Hypoiodite and Rate of Oxidation of Glucose in Alkaline Hypoiodite Solution at 23°.

Series No.	Glucose.	Time.	0.1 N iodine in form of:		0.1 M iodine consumed by glucose.	Iodine-hypoiodite in solution.	Hypoiodite converted to iodate-iodide.	Glucose oxidized.
			Iodine-hypoiodite.	Iodate-iodide				
	mg.	*min.*	*cc.*	*cc.*	*cc.*	*per cent*	*per cent*	*per cent*
I	90	2	1.45	10.00	8.55	12.7	87.3	85.5
		5	0.48	10.16	9.36	4.5	95.5	93.6
		8	0.42	10.16	9.42	4.0	96.0	94.2
		11	0.22	10.26	9.52	2.1	97.9	95.2
		15	0.12	10.27	9.61	1.1	98.9	96.1
		30	0.05	10.27	9.68	0.5	99.5	96.8
		120	None.	10.35	9.65	0.0	100.0	96.5
		240	"	10.35	9.65	0.0	100.0	96.5
II	90	2	3.10	7.60	9.30	29.0	71.0	93.0
		5	1.57	8.61	9.82	15.5	84.5	98.2
		8	0.84	9.21	9.95	8.4	91.6	99.5
		11	0.65	9.36	9.99	6.5	93.5	99.9
		15	0.64	9.38	9.98	6.4	93.6	99.8
		30	0.30	9.69	10.01	3.0	97.0	100.1
		120	0.07	9.93	10.00	0.7	99.3	100.0
		240	0.03	9.97	10.00	0.3	99.7	100.0

The method of procedure in Table III was as follows: To 10 cc. of 0.9 per cent glucose and 20 cc. (2 equivalents) of 0.1 N iodine-potassium iodide solutions were added 2 equivalents (40 cc. + 5 cc. to neutralize the gluconic acid formed) of 0.1 N sodium hydroxide, either immediately, as in Series I, or over an interval of 2 minutes at constant rate from a burette, as in Series II.   At the end of the time interval (tabulated in the third column) counted from the first addition of alkali, the analyses for "hypoiodite" and "iodate" were made as under Table II. The iodine consumed in the oxidation of glucose represents the sum of the hypoiodite-iodate titration subtracted from 20.   The percentages of hypoiodite and iodate are those calculated for iodine unconsumed by glucose.

A sufficiently high concentration of hypoiodite is possibly not attained when alkali is added immediately to a glucose-iodine solu-

tion for the reason that reactions (1) and (2) start out simultaneously. Under these conditions reaction (1), though it has a greater *initial* speed than reaction (2), will be overtaken by the second as the two near completion with the result that at this point the concentration of hypoiodite falls below the necessary optimum and both reactions end with the hypoiodite completely converted to iodate and the glucose in an incomplete state of oxidation. If, on the other hand, the alkali be added over an interval of 2 minutes, the major part of the hypoiodite will enter into the first reaction as it is formed, since the *initial* speed of the reaction glucose → gluconic acid is greater than that of the reaction hypoiodite → iodate. In other words, during the addition of the first increments of alkali the concentration of hypoiodite in the solution would be kept low, a condition unfavorable for the reaction hypoiodite → iodate.

A second interpretation might explain the phenomenon of the failure of quantitative oxidation when alkali is immediately added to glucose-iodine solutions; namely, the initial high concentration of hydroxyl ions should favor any process of emolization of glucose to unoxidizable ketose. If this be the case, then if glucose and alkali be first mixed and iodine be added to the mixture over an interval of 2 minutes, one should obtain 96 per cent oxidation. As a matter of fact, one obtains some 98 per cent oxidation. The discrepancy is even more striking if one permits glucose and 0.1 N alkali to stand 12 hours before adding iodine, for nearly 25 per cent of glucose will remain unaccounted for. It may be assumed therefore, that a small amount of glucose is actually converted to ketose under these conditions, and that the rapid conversion of hypoiodite to iodate does not completely explain the phenomenon of incomplete oxidation.

If potassium iodide be added in excess to iodine-glucose solutions (thus tending to increase the concentration of hypoiodite by repressing the reaction $3NaOI = NaIO_3 + 2NaI$), and then 2 equivalents of sodium hydroxide be added at one time, the oxidation progresses approximately to the same point as when iodine is added over an interval of 2 minutes to glucose-alkali solutions. This observation confirms the assumption that a high initial hydroxyl ion concentration hinders in part the reaction glucose → gluconic acid from going to completion despite a high hypoiodite concentration at the end of

the reaction. Whether this hindering effect can be attributed entirely to a process of enolization of the sugar, to an anticatalytic effect, or to some other remote cause is of course difficult to say when one deals with highly complex alkaline glucose-iodine solutions in which rapid concentration changes are taking place.

SUMMARY.

An explanation, based on the necessity of a proper hydroxyl ion concentration, for the stoichiometrical progression of the reaction glucose → gluconic acid in alkaline solutions of iodine, has been offered, and found to agree with the observed reaction curves.

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THE PREPARATION OF HEXONIC AND BIONIC ACIDS BY OXIDATION OF ALDOSES WITH BARIUM HYPOIODITE.

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INTRODUCTION.

In seeking a method for the conversion of an aldose to the corresponding monobasic sugar acid the chemist as a rule reverts to the classical principle used to such great advantage by Emil Fischer. As a reagent for bringing about this conversion bromine does not possess all the advantages which might be desired, for in some instances, in addition to the great period of time necessary for the oxidation to reach completion, it is difficult to control the conditions of reaction in a manner so as to yield an end-product free from the unchanged parent substance and from products of further oxidation.

During the course of an investigation (1) on the chemical nature of an aldobionic acid isolated as a hydrolytic product from the specific polysaccharide produced by Type III pneumococcus during growth, it became necessary to oxidize this aldehydic sugar acid as a step in the elucidation of its structure. For purposes of oxidation bromine failed. The present study found its impetus in this problem and has as its objective a general method having the advantages of good yields, freedom from too many technical difficulties, and finally in bringing about the conversion, aldose → hexonic acid, unfailingly and quantitatively.

A review of the various methods employed in the quantitative estimation of reducing sugars reveals only one in which the reaction between aldose and oxidant progresses stoichiometrically. The reaction $RCHO + 3NaOH + I_2 = RCOONa + 2NaI + 2H_2O$ was first employed in principle by Romijn (2), later by Colin and Liévin (3), and was finally shown to progress in a stoichiometric manner

under the conditions prescribed in the ingenious method of Willstätter and Schudel (4). It was thought possible to make use of the above reaction in devising a method for preparing hexonic and bionic acids. There is however a serious disadvantage in using potassium iodide-iodine and sodium hydroxide to carry out this oxidation, for it is obviously most difficult to rid the reaction product of these inorganic constituents. There is a second disadvantage in following implicitly the directions of Willstätter and Schudel in regard to the concentration of reagents; namely, the necessity of employing an enormous fluid bulk for the oxidation of a few gm. of material. To eliminate these two difficulties it was thought possible to substitute barium iodide-iodine and barium hydroxide for the reagents of Willstätter and Schudel and to carry out the oxidation in solutions of higher concentration. If the conditions of reaction be properly regulated it should still progress in the manner $2RCHO + 3Ba(OH)_2 + 2I_2 = (RCOO)_2Ba + 2BaI_2 + 4H_2O$ and one should ultimately obtain in the reaction mixture inorganic constituents which can be eliminated with the greatest ease, leaving in the mother liquid the desired sugar acid as end-product.

EXPERIMENTAL.

Methods.

In order to ascertain whether glucose could be quantitatively oxidized to gluconic acid analyses were made under the conditions described by Willstätter and Schudel but substituting 0.1 N barium hydroxide and 0.1 N iodine-barium iodide solutions for the usual reagents.

The method of procedure was as follows:

20 cc. of 0.1 N iodine-barium iodide solution (containing 25 gm. of barium iodide per liter) were added to 10 cc. of 0.9 per cent glucose solution and to the mixture were then added 45 cc. of 0.1 N barium hydroxide at constant rate from a burette. After standing for 15 minutes the solution was acidified with a slight excess of normal hydrochloric acid and the liberated iodine was titrated with 0.05 N sodium thiosulfate, using starch as indicator.

The results which are given in Table I show that barium hypoiodite is just as effective as is sodium hypoiodite in bringing about a quantitative oxidation of glucose to gluconic acid.

A second study was undertaken to ascertain whether glucose could be oxidized quantitatively in solutions of barium hypoiodite of higher concentration than decinormal, and whether the addition rate of barium hydroxide, as well as its final concentrations, would affect the ultimate outcome of the stoichiometry of the oxidation. Barium hydroxide of 0.4 N (approximately a saturated solution at room temperature) and 0.3 N iodine were chosen for this study. The analyses were carried out in the following manner.

90 mg. of solid crystalline glucose were added to 7 cc. of 0.3 N iodine-barium iodide (containing 75 gm. of barium iodide per liter). To this solution was then added the number of equivalents of barium hydroxide as indicated in the third column of Table II, plus 1.25 cc. to neutralize the gluconic acid formed, from a

TABLE I.

Relative Oxidizing Power of 0.1 N Iodine-Potassium Iodide and 0.1 N Iodine-Barium Iodide on Glucose in Alkaline Solutions.

Series I. Using 0.1 N potassium iodide-iodine and 2 equivalents of 0.1 N sodium hydroxide solutions.			Series II. Using 0.1 N barium iodide-iodine and 2 equivalents of 0.1 N barium hydroxide solutions.		
Glucose.	0.1 N iodine utilized.	Glucose oxidized.	Glucose.	0.1 N iodine utilized.	Glucose oxidized.
mg.	*cc.*	*per cent*	*mg.*	*cc.*	*per cent*
45	4.98	99.6	45	4.98	99.6
90	10.00	100.0	90	9.99	99.9
135	14.98	99.9	135	14.97	99.8

burette at constant rate over the interval of time indicated in the fourth column. The mixture was permitted to stand 15 minutes and was then titrated, after acidification with a slight excess of normal hydrochloric acid, with 0.05 N sodium thiosulfate solution, using starch as indicator. The barium hydroxide used in these experiments was prepared by recrystallizing Merck's reagent barium hydrate 8 times from water. A solution of this, when exactly neutralized with sulfuric acid and freed from barium sulfate, gave no visible residue on evaporation.

Sulfuric acid may not be used for acidification because not all of the iodine originally present can be accounted for. This disappearance of iodine is not entirely a phenomenon of adsorption for it was observed that the longer the solutions stood before acidification the greater was the loss of iodine. Since the formation of barium iodate takes place quickly in the solutions, this loss of iodine may be due to the formation of an insoluble barium-iodate-sulfate complex.

The results, as given in Table II, demonstrate that glucose is more than quantitatively oxidized to gluconic acid as measured by the iodine consumption, and that the base must be added to the iodine-glucose solution over an interval of time greater than 2 minutes in order to achieve such oxidation. It is seen, furthermore, that for oxidation to reach the end-point it is unnecessary to have more than 1 equivalent of base in the solution.

Although glucose is in part oxidized beyond the gluconic acid stage it was thought wise to choose the higher concentrations of

TABLE II.

Effect of Rate of Addition and Ultimate Concentrations of 0.4 N Barium Hydroxide on Oxidation of Crystalline Glucose Dissolved in 0.3 N Iodine-Barium Iodide Solution at 23°.

Glucose.	0.4 N Ba(OH)₂ added.	Equivalents of Ba(OH)₂.	Time over which Ba(OH)₂ was added.	0.3 N iodine utilized by glucose.	Glucose.
mg.	*cc.*		*min.*	*cc.*	*per cent*
90	6.25	1.00	3	3.55	106.5
90	7.81	1.25	3	3.54	106.2
90	9.10	1.50	3	3.55	106.5
90	11.25	2.0	Immediately.	2.00	66.0
90	11.25	2.0	2	3.15	94.5
90	11.25	2.0	3	3.54	106.2
90	11.25	2.0	4	3.56	106.8
90	38.75	6.0	4*	3.52	105.6

* First 2 equivalents were added over 3 minutes and the remaining 4 equivalents over 1 minute.

barium hydroxide and iodide-barium iodide as reagents for preparing sugar acids on a large scale, rather than decinormal solutions, because it was felt that the great economy of fluid bulk would offset the loss of end-product through processes of purification. Glucose, maltose, and lactose were subjected to oxidation, and the corresponding gluconic, maltobionic, and lactobionic acids were isolated as described below.

Preparation of Calcium Gluconate.

9.0 gm. of crystalline glucose were dissolved in 666 cc. of 0.3 N barium iodide-iodine[1] solution and to it while stirring was added 1 liter of 0.4 N barium hydroxide

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[1] Prepared by dissolving 75 gm. of Merck's barium iodide and 38.1 gm. of resublimed iodine in water and diluting to 1 liter.

at constant rate of flow over an interval of 3 minutes. The mixture was allowed to stand 15 minutes and was then acidified with 18.5 cc. of concentrated sulfuric acid dissolved in 150 cc. of water, after which an excess (150 gm.) of lead carbonate (Kahlbaum I) was immediately added. The mixture after rapid mechanical stirring, became neutral to Congo red paper. When this point was reached the precipitate was permitted to settle and the supernatant liquid, which contained lead gluconate, was evaporated *in vacuo*. The precipitate of lead carbonate, lead iodide, and barium sulfate was centrifuged and washed several times and the washings were added to the above supernatant liquid. The distillation *in vacuo* rids the solution of iodine. The concentrated aqueous solution of lead gluconate was filtered, the lead was precipitated with a slight excess of sulfuric acid, and the small traces of hydriodic acid still in solution were removed by the addition of a small amount of silver sulfate. After filtration of the silver iodide, the remaining silver and traces of lead in the filtrate were removed with hydrogen sulfide. One thus obtains a solution which should contain no inorganic constituent save sulfate ion which may be quantitatively removed by barium hydroxide. The final filtrate, which contained free gluconic acid, was boiled with an excess of calcium carbonate and a small amount of norit, and after cooling was filtered. The resulting solution of calcium gluconate was quite water-clear and colorless. It was evaporated *in vacuo* to a syrup and was poured into 10 volumes of redistilled methyl alcohol. 10.8 gm., or 91 per cent of the theory, of crude calcium gluconate were recovered. When recrystallized from water 8.2 gm. of crystalline calcium gluconate were obtained.

0.1002 gm. substance: 0.1234 gm. CO_2 and 0.0467 gm. H_2O.
0.1688 "   "   : 0.0219 " CaO.

	per cent	per cent	per cent
Calculated for $(C_6H_{11}O_7)_2Ca$.	C 33.47,	H 5.15,	Ca 9.31.
Found.	" 33.59,	" 5.21,	" 9.27.

Preparation of Calcium Maltobionate.

This product was prepared from 18 gm. of maltose exactly as was calcium gluconate. The crude product, which is also obtained in yields which vary between 80 and 90 per cent, always analyzes high in carbon and hydrogen, and low in calcium. In order to obtain a product which analyzes correctly the crude material is purified according to the method of Levene (5). This consists in dissolving the salt in approximately 3 times its weight of water, and precipitating in a centrifuge bottle with 1½ volumes of alcohol. About 50 to 60 per cent of salt is thus precipitated. The mixture is centrifuged and the supernatant liquid is poured off and saved for reworking, while the oily calcium maltobionate is dissolved in a small amount of water and precipitated by throwing into 10 to 15 volumes of methyl alcohol. Two different preparations were made in this manner.

Preparation 1.   0.0906 gm. substance: 0.1272 gm. CO_2 and 0.0456 gm. H_2O.
              0.1784 gm. substance: 0.0130 gm. CaO.

<div style="text-align:center">

per cent per cent per cent

Calculated for $(C_{12}H_{21}O_{12})_2Ca$.   C 38.18, H 5.6, Ca 5.31.

Found.   " 38.28, " 5.63, " 5.21.

</div>

Preparation 2.   0.1180 gm. substance: 0.1676 gm. CO_2 and 0.0600 gm. H_2O.

0.1153 "   "   : 0.0090 " CaO.

<div style="text-align:center">

per cent per cent per cent

Found.   C 38.73, H 5.68, Ca 5.55.

</div>

Preparation of Calcium Lactobionate.

This product was prepared and purified as was calcium maltobionate.

0.1178 gm. substance: 0.1663 gm. CO_2 and 0.0600 gm. H_2O.

0.1208 "   "   : 0.0089 " CaO.

<div style="text-align:center">

per cent per cent per cent

Found.   C 38.50, H 5.73, Ca 5.30.

</div>

All analytical samples were dried to constant weight *in vacuo* over successive baths of carbon tetrachloride, water, and toluene.

Dr. P. A. Levene was kind enough to suggest the method of purification of crude calcium maltobionate and lactobionate; without his suggestion this method would not have proven feasible.

It might be suggested that this method may be used in the preparation of acids of the methylated sugars, since Sobotka (6) has shown that they react stoichiometrically with sodium hypoiodite. In cases in which only 1 or 2 gm. of valuable material are to be subjected to oxidation by this method it is advisable to use solutions of decinormal strength.

CONCLUSIONS.

A method for the preparation of hexonic and bionic acids which makes use of barium hypoiodite as an oxidant has been described. This method possesses the advantage of speed, of ease in manipulation, of furnishing good yields, and of employing inorganic reagents which may ultimately be eliminated quickly and quantitatively, leaving in solution an end-product readily purified.

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THE OCCURRENCE OF DEGRADED PNEUMOCOCCI IN VIVO.

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It has been well demonstrated that under certain experimental conditions *in vitro* pneumococci undergo variations. It is of great importance to determine whether variants ever appear *in vivo* and if so, under what conditions. This is especially important since several investigations have suggested that recovery from pneumococcus infection may depend largely upon a change of virulent pneumococci into avirulent and phagocytable forms. This paper describes attempts to determine the experimental conditions favoring a change of virulent (S) pneumococci into avirulent (R) forms within the animal body.

It has been repeatedly shown (1–3) that by subjecting type-specific (S) pneumococcus strains to various unfavorable cultural environments *in vitro*, certain biological characteristics of the bacteria are profoundly affected. The changed bacteria no longer elaborate the specific carbohydrate element upon which type specificity depends and they are therefore agglutinable in heterologous as well as homologous antipneumococcus serum (4), they are no longer virulent for mice, and they are easily phagocyted (5, 6). These organisms have been designated R pneumococci by Griffith on account of the rough surface of the colonies which they form when grown on solid media, in contradistinction to the smooth surfaced colonies formed by S or typical virulent pneumococci. This difference renders the two forms distinguishable microscopically and often macroscopically when colonies of both forms are present on the same blood agar plate.

An intermediate form which is somewhat less virulent than the original S strain and also possesses slight serological differences has also been described (7). By animal passage it is possible to restore the original virulence and type specificity of this form. On the other hand, by placing it under unfavorable cultural environment the intermediate

form is easily converted into the R form. It would seem that the intermediate organisms represent a transitional stage between the S and the R forms.

Although they have been carefully searched for, R forms have not been encountered in the cultures of sputum or blood of patients, either during the period of pneumococcus infection or during convalescence, or in direct cultures from the lungs at autopsy. But in case the change from S to R does take place *in vivo* it seems probable that R forms would be phagocyted and destroyed as soon as they appear. Therefore, to determine the presence of the R forms *in vivo*, it would probably be necessary to employ a technic under which the infection would remain localized over a considerable period of time without killing the animal, and under which the free access of phagocytes to the bacteria would be inhibited. It was thought that these conditions might be obtained by embedding subcutaneously in the experimental animal an agar mass inoculated with a virulent strain of Pneumococcus.

Method.—15 cc. of melted nutrient agar was inoculated with 0.5 cc. of a young broth culture of Pneumococcus. A wire loop about 4 cm. in diameter was pressed firmly over a shaved area of skin of the anesthetized animal and the fluid agar injected subcutaneously into the area enclosed by the loop. Ice was applied until the agar had solidified into a firm hemispherical nodule. Observations were made from time to time by puncturing the focus with a hypodermic needle, aspirating some of the material, and plating it on blood agar plates. The plates were examined for S, R, and intermediate colonies after 12–18 hours incubation.

Experimental.

Agar Foci in Dogs.—Dogs were first employed since these animals have a natural relative immunity to Pneumococcus, and were likely to withstand infection over a considerable period of time. Two dogs were injected with agar containing Type I pneumococci according to the method described. Material was aspirated from the foci at intervals from the 1st to the 14th day after injection. But R forms never appeared on the culture plates made from the material at any time, although S forms were constantly present in large numbers. The dogs soon became ill and S pneumococci were recovered from the blood stream on several occasions. In a few days abscesses formed at the site of injection in both animals and by the 10th day the skin

over the foci ruptured and thick pus was discharged. About the 14th day the lesions began to heal, the pneumococci disappeared and the dogs recovered. After a rest period of 2 weeks the same dogs, which were now assumed to have an additional degree of immunity, although specific agglutinins were not demonstrable in the blood serum, were reinjected with infected agar. A repetition of the previous events occurred. The dogs recovered and no R forms were encountered either from the foci or from the blood stream. While it was impossible with this technic to demonstrate the appearance of R forms in dogs, it should be mentioned that 11 years ago Bull (8) discovered that certain changes may be undergone by pneumococci during the course of experimental septicemia in dogs. In one dog which lived for 10 days before dying with meningitis, pneumococci isolated from the blood on the 9th day "grew in chains and were non-virulent."

Agar Foci in Rabbits.—The same technic was then employed with rabbits instead of dogs, and instead of using a Type I strain, a Type III strain was employed. This type was used because rabbits have a high degree of resistance to Type III pneumococci, in contrast to their susceptibility to Types I and II, and it was thought that animals infected with this type would live longer and the bacteria would therefore have a greater chance to undergo variations. The special strains employed have been shown to readily undergo variations *in vitro*. Three rabbits were used. Daily examination of the foci by aspirating and plating material from the foci on blood agar plates showed that only S forms were present. The number of pneumococci gradually diminished until no more were demonstrable by the 6th day after injection.

Agar Foci in Guinea Pigs.—In a similar manner six guinea pigs, actively immunized against Type I pneumococci, were injected with agar containing Type I pneumococci. Repeated cultures from the foci showed the presence of S forms alone until the 5th day after injection when eight R colonies were found on a blood agar plate seeded from the focus of one guinea pig. R colonies were subsequently recovered from the foci of the other five guinea pigs at intervals up to 6 weeks after injection. In all instances however, the S colonies greatly outnumbered the R forms and were recovered from the foci for 6 weeks, or until the agar was absorbed. Normal (unimmunized)

guinea pigs were also tested but died from pneumococcus septicemia too soon to be suitable for the experiment.

In spite of the mass of agar surrounding the pneumococci in the subcutaneous foci, the object of preventing the access of phagocytes was not attained. Stained films made from the aspirated agar showed the constant presence throughout the agar mass of many phagocytes containing pneumococci.

As mentioned above, it was assumed that the R forms, being easily phagocyted, were rapidly destroyed or removed from the site of infection. To test this assumption, agar masses inoculated with a heavy suspension of R pneumococci were injected subcutaneously into two guinea pigs. It was found that the R forms were recoverable for 5 weeks after injection or until the foci had been completely absorbed. Although microscopic examination revealed the presence of great numbers of phagocytes, they apparently had but little influence in removing the R forms as long as the focus remained.

In order to eliminate the activity of phagocytes entirely, the technic was then modified as follows: Agar, which was inoculated with S pneumococci, was enclosed in a glass vial sealed with a collodion membrane. The membrane was an effective barrier against phagocytes but permitted the diffusion of fluids.

Method.—5 cc. wide mouth vials were filled, nearly to the top, with melted agar and the mouths were covered with thin muslin caps. After sterilization the agar was inoculated with 0.1 cc. of pneumococcus broth culture by means of a long hypodermic needle, and the muslin-covered mouths were dipped in a thin solution of collodion. The vials were then immersed in 95 per cent alcohol for several minutes to render the membrane more permeable. The sealed vials were embedded subcutaneously in rabbits. Repeated observations were made by inserting a long hypodermic needle through the skin of the animal and the membrane of the vial, aspirating some of the agar, and plating it on blood agar.

Control vials were prepared in the same manner but, instead of being placed *in vivo*, they were immersed in normal rabbit serum in large test-tubes. The tubes were kept in the incubator at 39°C. which was considered to be the average temperature of the vials while embedded in the rabbits. The serum was changed weekly.

Vials containing agar inoculated with cultures derived from a single diplococcus of a Type I strain were inserted subcutaneously in eight normal and three passively immunized rabbits and allowed to remain

for several weeks. Eight vials similarly prepared were immersed in normal serum and kept in the incubator as controls. Agar was aspirated from the vials and plated on blood agar at intervals of from 1 to 5 days. Several of the normal (unimmunized) rabbits soon died from pneumococcus septicemia due to infection resulting from leakage of the vials after aspiration. The unimmunized rabbits which survived were observed over a period of 8 weeks. Their serum did not show the presence of agglutinins for Type I pneumococci even at the end of this time.

R colonies were invariably found on plates made from all of the vials embedded in rabbits. They appeared, often with the intermediate forms, as early as the 3rd day after inoculation and were recoverable up to 8 weeks. The variant colonies were usually outnumbered by the S forms and in no instance were the S forms entirely replaced by them. It was rather surprising to find that both R and intermediate colonies were derived from the agar vials in normal and immunized animals alike.

The eight control vials inoculated at the same time with the same culture of Type I pneumococci were kept in the incubator. R colonies never appeared on plates made from these vials although the S forms remained during the period of observation.

Experiment with Type III Pneumococcus.—Three agar vials were inoculated with a Type III pneumococcus culture obtained from a single diplococcus from the blood culture of a pneumonia patient, and embedded subcutaneously in three rabbits. Frequent plating of the agar at intervals during a period of 5 weeks never revealed the presence of R or intermediate forms although S forms remained viable throughout.

This culture proved to be a strain especially refractory to modification as was determined by subjecting it to treatment *in vitro* by methods which invariably caused the appearance of R forms in other strains. Frequent search during 40 transfers in broth containing 5 per cent Type III antipneumococcus serum, or during 11 transfers in broth containing optochin yeast (9), or during 53 transfers on blood agar plates, did not reveal the presence of any R or intermediate forms. The culture remained unchanged in virulence and type specificity. Takami (10) has also encountered resistant strains of this

nature. He found that, of 28 strains, 12 never gave rise to variant forms even after prolonged subculture on blood agar.

Spontaneous Appearance of R Forms in Vivo.—Aside from the experimental production of the variant forms it is of interest to report the spontaneous occurrence of R forms *in vivo*. Through the courtesy of Dr. Mary B. Kirkbride of the New York State Department of Health, it was learned that atypical pneumococci were occasionally recovered from the blood stream of horses during the process of immunization with live virulent cultures for the production of antipneumococcus serum. The atypical strains were recovered from 6 to 16 months after the horses were first inoculated with a Type I pneumococcus culture. During this time the horses had a low grade fever, anorexia, loss of weight, rapid irregular pulse, heart murmurs, swelling and stiffness of the joints. The animals finally died and autopsy revealed an endocarditis present in all. Atypical pneumococcus strains from four of these horses were obtained and examined in this laboratory. They proved to have many characteristics in common with the variant or R strains previously studied. Each of the four strains produced colonies which differed slightly morphologically from one another and were distinguishable on blood agar plates. It was previously noted that in a number of instances, R colonies differing from one another in appearance were also recovered from the agar vials embedded in rabbits. The four horse R strains together with four strains derived from R colonies from the rabbit vials were tested for variations in virulence and agglutinability. All strains failed to kill mice in doses of 1 cc. of a young broth culture, were bile-soluble, and were serologically alike. The morphological colony differences between the various strains persisted even after repeated plating. Repeated (six) animal passages of the R strains failed to restore either virulence or specificity.

DISCUSSION AND CONCLUSION.

It is conceivable that a change from the virulent, non-phagocytable S form of Pneumococcus to the avirulent phagocytable R form may take place in pneumococcus disease, but the experiments here reported do not settle the question whether or not this is an important factor in determining the outcome in natural infection. It has been shown

experimentally that the degradation from the S form to the R form actually does take place in cultures of Pneumococcus growing in agar subcutaneously embedded in guinea pigs, in agar enclosed in vials subcutaneously embedded in rabbits, and spontaneously in the blood stream of infected horses. However, it was not possible in any of the experiments here cited to demonstrate the complete change from S to R pneumococci before the bacteria disappeared from the body. When the intermediate or R forms did appear, they were always accompanied and usually exceeded in number by the S forms and all three forms disappeared together. S organisms may disappear entirely without evidence of first going through the intermediate and R stages. On the other hand, contrary to expectations, pure cultures of R forms remained viable in subcutaneous foci for weeks although apparently freely accessible to the action of phagocytes. It seems of some significance that the R forms appeared early in the vials (inoculated with S pneumococci) in immunized and normal rabbits alike, indicating that the presence of demonstrable specific immune bodies was not alone responsible for the variation of the bacteria.

Of some importance also is the fact that R forms were never derived from similarly prepared control cultures growing *in vitro* at the same temperature and immersed in normal serum, although the S forms remained viable and unaltered for 6 weeks. It is likely that variations of pneumococci do not occur readily when S cultures are exposed to normal serum *in vitro*, especially when growing in closed vials under a diminished oxygen supply, for it has previously been shown (2) that only slight variation occurs even after prolonged (240) transfers in heterologous serum broth in the test-tube. It is possible, therefore, that the variation which occurred among pneumococci growing in agar vials embedded in normal rabbits was actually provoked by unknown influences in the living tissue fluids.

Although R forms have been shown to occur *in vivo*, no positive evidence can be derived from these experiments to prove that recovery from pneumococcus infection depends upon the degradation of the virulent S forms of pneumococci to the avirulent R forms and the subsequent destruction of the latter by phagocytes.

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OXYGEN POISONING IN MAMMALS.

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PLATES 27 AND 28.

INTRODUCTION.

The increasing use of oxygen in clinical medicine would seem to make worth while a study of the toxic effects which may result from its prolonged inhalation in high concentrations. The observation that oxygen inhalation may lead to pathological states is not a new one. And yet it has seemed important to us, because of our own constant use of oxygen inhalation as a therapeutic measure, to investigate this problem further, giving particular attention to the nature of the injury and the mechanism of death. The fact that many of the experiments done in the past were performed on animals housed in small containers with inadequate ventilation made it appear especially desirable to repeat experiments under conditions in which CO_2 removal, temperature, humidity and air motion were controlled, and to rule out definitely the possibility of death resulting from respiratory infections so prone to occur in caged animals and in rabbits in particular. It seemed not improbable that some of the pulmonary lesions described in the literature might be the result of respiratory infections perhaps resulting from a lowered resistance of lung tissue when exposed to an abnormal mixture of atmospheric gases.

Lavoisier is said to have remarked the poisonous effects of oxygen inhalation shortly after the discovery of this gas in the atmosphere. Regnault and Reiset (2) in 1849 demonstrated that the respiration of different species of animals in an atmosphere containing two or three times the normal concentration of oxygen remains unchanged. They found no alteration in the amount of oxygen consumed, in the respiratory quotient, or in the general behavior of the exposed animals. No further investigation in this field appears to have been made until the

classical experiments of Paul Bert (3) established the fact that oxygen at high tensions is a powerful poison.   Bert showed that sparrows exposed to atmospheric air at 15 to 20 atmospheres pressure developed convulsions and died.   If pure oxygen were used in the compression chamber only one-fifth as much pressure was needed to produce the same effects.   On the other hand, if oxygen-poor air were used in the compression chamber the sparrows survived until overcome by the accumulated CO_2.   Bert concluded that the toxicity of highly compressed air was due to the high tension of the contained oxygen, which he believed exerted a direct toxic effect upon the central nervous system.   He was unable to reproduce the symptoms by the injection into a normal dog of a large amount of blood taken from a dog in oxygen convulsions.   From this he argued that the status epilepticus which he described was not the result of a circulating poison secondarily produced by the oxygen.   He found that high oxygen tensions had a similar effect on other laboratory animals and was also injurious to insects, arachnids, myriapods, molluscs, earthworms and germinating seeds.   It inhibited the putrefaction of meat and delayed the souring of milk.

20 years later the problem was taken up by Lorrain Smith (4), who was the first to test out the effect of prolonged exposures to moderately high oxygen tensions. He found that mice suffered no ill effect from exposure for 8 days to 41.6 per cent of an atmosphere of oxygen, but that with 70 to 80 per cent of an atmosphere some mice died in 4 days of "congestion and consolidation" of the lungs, while others survived.   At 1.14 to 1.50 atmospheres of oxygen all the mice died of consolidation of the lungs in 40 to 70 hours.   At 1.66 to 1.89 atmospheres of oxygen mice, guinea pigs and larks died at 7 to 27 hours, the lung changes being similar.   At 3.55 to 3.57 atmospheres of oxygen mice died in 5 hours.   Mice which had begun to show dyspnea on exposure to this tension of oxygen died at once on being taken out of the chamber.   Two larks exposed to 3.017 atmospheres of oxygen developed convulsions in 10 minutes.   They were taken out of the chamber after 2 hours but both died.   There was nothing noteworthy in their postmortem appearances. Lorrain Smith showed that by a previous short exposure to a high oxygen tension or by raising the oxygen tension very slowly the exposure could be carried distinctly beyond the point which usually produces convulsions.   He also aimed to show that the toxic effect of oxygen is related to its tension in the inspired air and not its quantity in the blood.   In a chamber containing 0.4 per cent carbon monoxide as well as 3 atmospheres of oxygen larks developed convulsions as usual, although the arterial blood at the end of the experiment was only 38 per cent saturated with oxygen.

In brief, Lorrain Smith demonstrated that moderately high tensions of oxygen produce an inflammation of the lungs, while very high tensions have an irritating effect on the central nervous system.   He believed that the inflammatory reaction of the lung protected the central nervous system from pathological changes through interference with the diffusion of oxygen into the tissues.   There is considerable variation between species and between individuals of the same

species in their susceptibility to oxygen. The minimum tension necessary to produce stimulation of the central nervous system is always well above that required to bring about pulmonary inflammation, but if the tension is sufficient the nervous symptoms develop rapidly and death may occur before there are any demonstrable changes in the lungs.

In 1903 Hill and McLeod (5) considered the question of the effect of compressed air on the respiratory exchange. From their experiments on mice they concluded, in agreement with an observation of Paul Bert, that a partial pressure of oxygen equal to 1 atmosphere does not increase, but rather lessens the processes of oxidation.

On the other hand, experiments in lower animal forms (6) have shown that the metabolism of many lower invertebrates, both marine and terrestrial, and some of the higher invertebrates such as the lobster and annelid worm is proportional over a wide range to the oxygen tension. This does not appear to be true of vertebrates.

Curiously enough, Lavoisier's original observation of the toxic effects of breathing high concentrations of oxygen at atmospheric pressure did not receive the attention of later workers (with the exception of Lorrain Smith) until Schmiedehausen (1) and David (7) confirmed his findings. They reported that pure oxygen supplied to a dog through a tracheal cannula produced a mild pulmonary hyperemia in as short a period as 15 minutes. 1 hour's exposure of a dog to 90 per cent oxygen in a chamber brought about definite hyperemia and extravasation of blood into the bronchi and alveoli. Similar results were noted in mice and guinea pigs, particularly after longer exposures. One guinea pig was exposed to 40 to 60 per cent oxygen at atmospheric pressure for 69 hours and 37 minutes. 6 hours after being taken out of the chamber into room air the animal died. Postmortem examination showed bronchopneumonia. This is the only case we have found recorded in the literature of pulmonary inflammation following exposure to increased oxygen tensions of less than 70 per cent of an atmosphere.

Benedict and Higgins (9), in 1911, carried out a series of experiments on the effect of inhalation of oxygen-rich mixtures for short periods of time on normal young men. With 40, 60 and 90 per cent oxygen they found no change in metabolism or respiration but a definite decrease in the pulse rate, which was more or less proportional to the percentage of oxygen breathed.

Bornstein and Stroink (10), in 1912, report the first case of experimental oxygen poisoning in man. Bornstein placed himself in a pressure chamber exposed to 2 atmospheres of pure oxygen. After 50 minutes he began to have cramps, first in the right, then in the left arm. The cramps ceased as soon as the pressure was lowered. These investigators also carried out a series of experiments on the effect of high oxygen tensions on dogs, apes, cats and rats. A dog and an ape, kept for several months in 0.6 atmosphere of oxygen, showed slight anemia,—an interesting observation in view of the opposite effect of low oxygen tensions.

Retzlaff (11), in 1913, found that the inhalation of oxygen produces vasoconstriction of the pulmonary blood vessels in the cat, and he suggests that the

beneficial effect of oxygen administration in cardiac failure with pulmonary edema may be ascribed to the improved pulmonary circulation thereby produced.

In 1916, Karsner (12) made an extensive study of the pathology of oxygen poisoning in rabbits, and recorded the time relationships of the various changes and gave a definition of the type of pneumonia occurring. He found that "80 to 96 per cent oxygen under normal barometric pressure produces in 24 hours, or more commonly 48 hours, congestion, edema, epithelial degeneration and desquamation, fibrin formation, and, finally, a pneumonia, probably of irritative origin," and described by him as a "fibrinous bronchopneumonia." He also found a certain degree of congestion in all of the abdominal organs which he believed to be secondary to the damage done to the pulmonary circulation. There were no demonstrable changes in the hematopoietic system other than congestion.

In a later work (13) Karsner and his coworkers showed that high partial pressures of oxygen are definitely inhibitory to the growth of certain strains of bacteria, while on other strains they may have no effect. The growth of pneumococcus was not inhibited by high oxygen tension.

Cleveland (14), in 1925, reported a series of ingenious experiments which indicated that oxygen in high concentrations is peculiarly toxic to intestinal protozoa. In cockroaches, for instance, the oxygen is 135 times as toxic for flagellates and 26 times as toxic for ciliates as for the host cockroaches. Similar effects are seen in termites and to a less marked degree in frogs, salamanders and goldfish, so that the intestinal tracts of these animals can be cleared of protozoa by simply exposing them to a sufficient oxygen tension. This test is impossible to carry out in warm blooded animals because the host is more susceptible than the parasite.

Barach (15), in 1926, published a series of carefully controlled experiments on the effect of oxygen-rich atmospheres on normal rabbits and on rabbits with pulmonary tuberculosis. He found that 60 per cent oxygen produced no effect on the general appearance, activity and weight or growth of normal rabbits over periods as long as 1 to 4 months. Furthermore, no gross or microscopic pathological change was observed for periods as long as 1 to 2 months. Attempts to increase the resistance of the pulmonary epithelium to atmospheres containing 80 to 85 per cent oxygen by previous exposure to lower concentrations were unsuccessful. In one case pulmonary edema followed the inhalation of 70 per cent oxygen for 12 days. On the basis of these observations Barach states that the highest concentration of oxygen compatible with safety for therapeutic use should be regarded as 60 per cent.

EXPERIMENTAL.

Our own studies on the toxic effects of oxygen began with observations on three dogs and three rabbits kept in an atmosphere of approximately 80 per cent oxygen. The animals were placed in cages in a large oxygen chamber used ordinarily for the treatment of pneumonia patients. In this chamber it was possible to maintain satisfac-

tory cleanliness and isolation of one animal from the other and to provide for the proper removal of carbon dioxide and moisture, and maintenance of fairly constant conditions of temperature, relative humidity and air motion. Daily observations of the weight, body temperature and behavior of the animals, and more frequent records of the room temperature, humidity and oxygen concentration were made. After a 4 day control period with the chamber open to the atmospheric air the doors were closed and oxygen was admitted into the chamber. The oxygen content of the chamber was raised at once to a little over 50 per cent of 1 atmosphere and then gradually, during the next 30 hours, to 80 per cent, where, with minor fluctuations, it was maintained throughout the remainder of the experimental period.

Experiments on Dogs.

The first abnormal sign noted in the dogs was the refusal of food. This was observed on the 3rd day after exposure to 70 to 80 per cent oxygen in Dog 1, a young, immature dog, on the 4th day in Dog 2, and on the 5th day in Dog 3. All of the dogs lost weight after the 3rd day. *Vomiting* occurred on the 5th day in Dog 1, on the 6th day in Dog 3 and was absent in Dog 2. *Drowsiness* was first recorded on the 6th day in all three dogs. *Labored breathing* was first noticed on the 6th day in Dog 3, on the 7th day in Dog 1 and was absent throughout in Dog 2. This respiratory distress became progressively worse during the rest of the experimental period. It had the character of slow, labored, deep breathing often associated with an apparent expiratory effort. There were no seemingly significant variations in the body temperature. Text-fig. 1 shows the variations in body weight and temperature in relation to O_2 and CO_2 content of the chamber.

After 8 days exposure to 70 to 80 per cent of an atmosphere of oxygen the dogs were removed from the chamber and samples of their arterial blood were drawn for oxygen analysis by puncturing the femoral arteries.

Dog 1 was markedly cyanosed. There was a cardiac arhythmia, shown by electrocardiograph to be the result of extrasystoles. Blood-stained froth was pouring from the mouth. Breathing was labored. At each inspiration the flanks were sucked in and expirations were grunting. When turned on its back respiratory distress became very great. Analysis of the arterial blood showed an oxygen saturation of only 40 per cent.

Dog 2 showed no evidence of anoxemia or respiratory distress. The arterial blood was 94.6 per cent saturated with oxygen.

Dog 3, like Dog 1, though far less markedly, manifested respiratory disturbance. Respirations were slow and labored. The flanks sucked in with inspiration and expiration was accompanied by a prolonged groan. There was, however, no definite arterial anoxemia, the blood being 93.7 per cent saturated with oxygen.

The three dogs were then immediately and painlessly killed by the intravenous injection of from 10 to 20 cc. of a saturated solution of magnesium sulfate. This
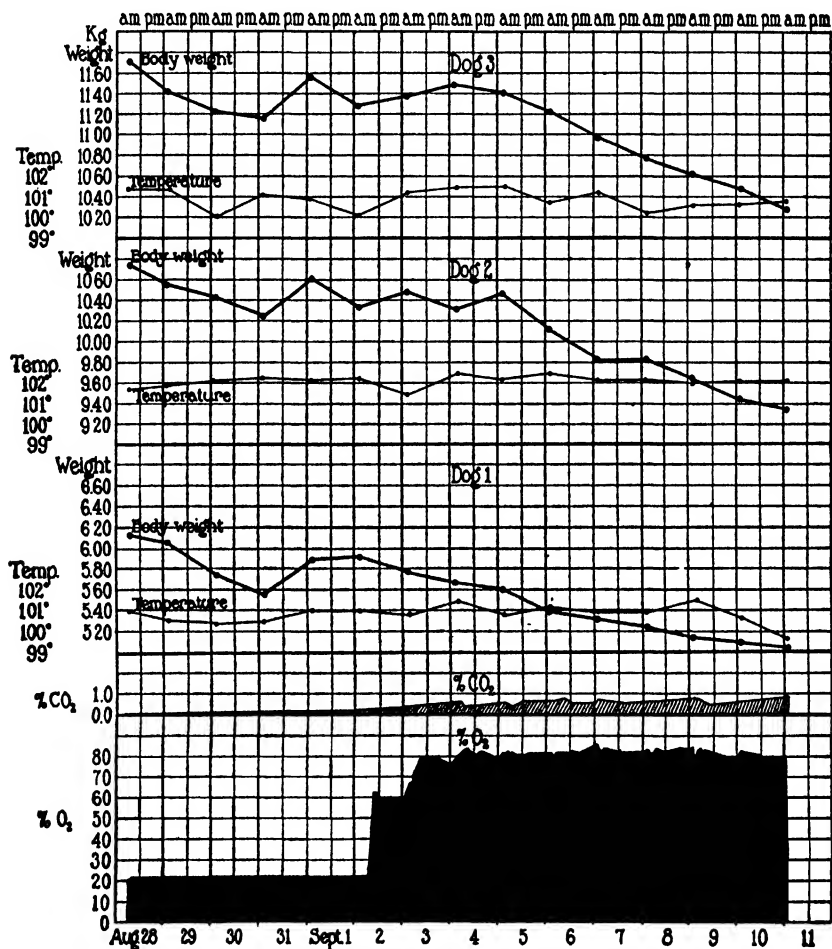
TEXT-FIG. 1. Chart showing loss of weight of three dogs confined in chamber containing 80 per cent oxygen. The oxygen concentration is represented by the black area; the carbon dioxide by the narrow shaded area. Carbon dioxide concentration remained below 0.8 per cent throughout the experimental period.

method is admirable for the study of pulmonary pathology, as the heart beat and respirations are arrested simultaneously and at once, unaccompanied by an ante-mortem struggle.

Autopsies on Dogs.

The gross and microscopic appearance of the lungs in the three dogs showed changes in keeping with the severity of their response to oxygen.   When examined by a pathologist* who was unfamiliar with the symptomatic course of these three animals the lesions were graded in order of intensity thus: Dog 1, Dog 3, Dog 2, which will be seen to correspond to the manifestations cited above.

Description of Lesions in Lungs of Dog 1.

When the thorax was opened the lungs were found to be collapsed.   They were mottled, beefy red—bright in some areas and dull red in others.   There were no normally pink lobes.   The trachea was full of blood-tinged froth.   There was no free fluid in the pleural cavities.   The lungs were disturbed as little as possible, except for punctures made for the purpose of making cultures.   They were fixed *in situ* by distending them with Helly's fluid injected through a tube tied in the trachea.   The roots were then ligated and the lungs carefully removed from the thorax and placed in Helly's fluid.   Histological preparations were made by the usual paraffin technique, sections being cut at 7 μ and stained with Giemsa and hematoxylin and eosin.   The sections revealed a general destructive process of a non-infectious character involving all parts of both lungs, the chief features being: (1) capillary engorgement with some hemorrhage; (2) the presence of interstitial and intraalveolar serum; (3) hypertrophy and desquamation of alveolar cells; and (4) interstitial and intraalveolar infiltration of mononuclear cells.

No microorganisms were seen in any of the sections examined.   It will be shown later that these lesions, though not so marked in the dog, are pronounced in the lungs of the rabbits exposed to oxygen in the same chamber with the dogs.   A photograph of a characteristic section of lung tissue from Dog 1 is shown in **Figs.** 1 to 3.

Description of Lesions in Lungs of Dog 2.

On opening the chest wall the lungs were collapsed and of essentially normal appearance, being uniformly coral-pink.   There was no evidence of infiltration or consolidation; nor was there any free fluid in the pleural cavities.   The lungs were treated in the same manner as those of Dog 1.   Microscopic examination showed little change except for an apparent capillary engorgement.

Description of Lesions in Lungs of Dog 3.

Both lungs were collapsed.   The right lung was, for the most part, normally pink in color, but there were a few small hemorrhagic areas near the periphery, especially on the anterior borders of the ventral lobe.   The left lung had several small hemorrhagic areas on the surfaces of all lobes which were otherwise of normal

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* Dr. L. T. Webster of the Staff of The Rockefeller Institute, whom we wish to thank for his help in interpreting the nature of the lesions.

appearance. There was no free fluid in the pleural cavities. Microscopic examination showed capillary engorgement with some hemorrhage. Fig. 4 shows a photomicrograph of a characteristic region taken from the right lower lobe of Dog 3.

The gross appearance of all the other viscera was essentially normal.

Attempts at Cultivation of Bacteria.

Broth cultures taken from the heart's blood in all three dogs were sterile, and material from the lung punctures streaked on blood agar also showed no bacterial growth.

Experiments on Rabbits.

Three rabbits, housed in separate cages, were placed in the same chamber with the dogs just described, and exposed to identical atmospheric conditions as the dogs. Daily observations of body temperature and weight were made as well as careful inspection of behavior. No significant fluctuation in temperature occurred. All of the rabbits showed a slight loss of weight during the experimental period. *The first symptom of significance was marked dilatation of the alæ nasi accompanying respiration.* This was noticed on the 6th day after exposure to 70 to 80 per cent oxygen in Rabbit 1; on the 6th day in Rabbit 2; on the 5th day in Rabbit 3. From their general appearance at this time the prediction was made that they would die in the order in which they eventually did die, namely No. 3 first, then No. 1 and finally No. 2. The dilatation of alæ nasi was followed by definite signs of respiratory distress, gasping inspirations with the use of the accessory muscles of respirations, the mouth opening wide with each inspiration. This was accompanied by definite cyanosis of the tissues about the nose and of the ear tips. These symptoms appeared from the 6th to 7th day in all three animals. The intensity of the respiratory distress grew worse, the animals showing marked orthopnea when turned on their backs for the purpose of taking their temperatures. Rabbits 1 and 3 died on the 7th day after exposure to 70 to 80 per cent oxygen.

From the appearance of cyanosis and the low oxygen saturation found in the arterial blood of Dog 1 it was believed that the immediate cause of death was probably anoxemia resulting from the lesion of the respiratory membrane. To test this point further it was decided to take Rabbit 2 out of the chamber, with the anticipation of its being immediately asphyxiated by the sudden lowering of alveolar oxygen tension. When this was done the animal at once had a convulsive seizure and died in a few moments after exposure to atmospheric air.

Autopsies on Rabbits.

With minor variations in intensity of reaction the gross and microscopic appearance of the three rabbits' lungs were closely similar. All three showed mottled dull to beefy red discoloration—with much gross edema, and either froth or fluid in the trachea. Rabbit 1 had 2 to 3 cc. of blood-tinged fluid in both pleural cavi-

ties, which, on direct smear, showed many lymphocytes and leucocytes and fibrin, but no bacteria.   A few cc. of a similar but not blood-tinged fluid was found in the pleural cavities of Rabbit 2.   None was seen in Rabbit 3.

Microscopic examination of the lungs of the three rabbits showed in varying degrees of severity the same reaction as seen in Dog 1, though on the whole a more intense one, *viz.:* capillary engorgement with hemorrhage, interstitial and intra-alveolar serum, hypertrophy and desquamation of alveolar cells, interstitial and intraalveolar infiltration of mononuclear and of a few eosinophilic cells.   No microorganisms were found in any of the sections examined.   Photomicrographs of sections from the lungs of Rabbits 1 and 3 are shown in Figs. 5 and 6.   Heart's blood cultures were sterile.   With the exception of an occasional mold and colony of large spore-bearing bacillus most of the plates streaked with material obtained from lung puncture were likewise sterile.

Experiment on a Guinea Pig.

Protocol.—December 3.   A guinea pig was placed under a bell jar, so arranged on a wooden platform that oxygen could be blown into the jar at the desired rate. A basket containing soda-lime, for the removal of CO_2, was suspended in the jar. At 4.25 p.m. the flow of oxygen through the jar was begun at the rate of 1 liter a minute.

December 4.   The guinea pig looks normal.   There is no dyspnea.   Analysis of sample of gas in the bell jar: Oxygen, 99 per cent.   Carbon dioxide, 0.67 per cent.

December 6.   12 noon.   Respirations deep.

5 p.m.   No apparent dyspnea.   The guinea pig has eaten lettuce and some oats.

December 7.   9 a.m.   Found dead.   Autopsy showed the lungs to be a deep, dull red, resembling liver in appearance.   Sections of the lungs placed in 10 per cent formalin sank to the bottom of the container.

Experiments on Mice.

Four full grown mice were placed by pairs into two dialyzing jars which were found to be convenient receptacles for subjecting them to the desired gas mixtures. The jars were partly filled with wood shavings to serve as bedding for the mice and provide for their warmth.   Two of the mice acted as controls and two of them were to be subjected to a high concentration of oxygen.   Compressed air was run into the control jar from a pressure tank at a rate of 1 liter per minute and oxygen into the other.   This rate of flow provided for an adequate removal of CO_2 and mois-ture.   It was believed that any harmful influence which might result from the effect of compression and commercial handling of oxygen might be controlled by the use of air compressed to the same degree in cylinders identical to those con-taining the oxygen.

Protocol.

Oxygen Mice.

Mouse marked with picric acid. Weight 19.6 gm.

Mouse marked with carbolfuchsin. Weight 20 gm.

Nov. 16, '25, 4.30 p.m. O_2 flow started at 1 liter per min.

Nov. 17, '25. Mice appear normal. O_2 concentration in jar 98 per cent.

Nov. 19, '25. Mice appear normal. They are lively and restless. They remain on top of the shavings, whereas control mice are buried.
O_2 concentration in jar 98 per cent. CO_2 0.39 per cent.

Nov. 20, '25. Mice appear normal. O_2 concentration 98.5 per cent.

Nov. 21, '25. Mice appear normal.

Nov. 22, '25, 11.30 a.m. Mouse marked with picric acid is found dead. Weight 15.8 gm. Mouse marked with carbolfuchsin is gasping for breath. Its mouth opens wide and its whole body shakes at each inspiration. Cyanosis of tail, feet and nose. 2.45 p.m. Mouse dead. Weight 15.3 gm.

Control Mice. Weight 25 gm.

Mouse marked with mercurochrome. Weight 25 gm.

Unmarked mouse. Weight 19 gm.

Nov. 16, '25, 4.30 p.m. Air flow started at 1 liter per min.

Nov. 17, '25. Mice active. Buried in shavings most of the time. No condensation of moisture on walls of jar. Analysis of air in jar: O_2 = 22.09 per cent. CO_2 = 0.22 per cent.

Nov. 18, '25. Mice appear normal. Analysis of air in jar: O_2 = 21.58 per cent. CO_2 = 0.25 per cent.

Nov. 21, '25. Mice appear normal.

Nov. 22, '25. Mice appear normal. Analysis of air in jar: O_2 = 21.68 per cent. CO_2 = 0.4 per cent.
4.30 p.m. Compressed air tank nearly empty. Jar connected with house air pressure system and air run in at the same rate of flow, *viz.:* 1 liter per min.

Nov. 23, '25. Mice appear normal.

Nov. 24, '25. Mice appear normal.

Nov. 25, '25. Mice appear normal. Mouse marked with mercurochrome now weighs 27.6 gm. Unmarked mouse now weighs 20.6 gm. Jar cleaned and fresh wood shavings put in. Flow from compressed air cylinder again begun at rate of 1 liter per min.

Nov. 27, '25. Mice appear normal.

Nov. 28, '25, 8.40 p.m. Mice appear normal.

Nov. 29, '25, 10.30 a.m. Mice appear normal.

Nov. 30, '25. Mice removed from jar. They are active and normal in appearance. Mouse marked with mercurochrome weighs 27.1 gm. Unmarked mouse weighs 20.5 gm.

Autopsy of both these mice showed the lungs to be uniformly discolored a deep red, almost indistinguishable from liver. The lungs sank when placed in Zenker's fluid.

The experiments thus far reported demonstrate quite clearly, we believe, that for several mammalian species, the mouse, guinea pig, rabbit and dog, inhalation of commercial oxygen in concentrations greater than 70 per cent of an atmosphere may lead to a train of physiological changes consisting of drowsiness, loss of appetite, loss of weight, dyspnea and cyanosis, usually culminating in death from extreme oxygen want. The cause of the oxygen want is undoubtedly to be sought for in the acute pulmonary changes, to be characterized as a diffuse hemorrhagic edema of the lungs found in all these species.

Is it possible that this destructive process is due not to the oxygen itself but to some impurity in it introduced in the process of manufacture? The oxygen used thus far in this work was commercially prepared by a method now commonly employed, namely: the fractional distillation of liquid air. Before concluding that the pathological effect is due to oxygen and not to the presence of impurities, parallel experiments were done on mice kept in jars containing oxygen prepared in two differing ways: (1) by the so called "air reduction" process, (2) by the electrolytic dissociation of water.

This experiment shows that mice confined in a jar containing 96 to 98 per cent oxygen electrolytically prepared show the same physiological and pathological reactions as mice confined in equivalent concentrations of oxygen prepared by the "air reduction" process.

Burrows (16) reported the observation that oxygen was inhibitory to the growth of tissue cultures only when the gas had been led through rubber tubing, and believed that the toxic effect was due to a substance formed by the reaction of oxygen or ozone with rubber. Burrows, however, prepared his oxygen by the electrolysis of dilute H_2SO_4. By this method there is usually a contamination with ozone, which Burrows removed by bubbling the gas through olive oil. Under these circumstances it was not inhibitory to the growth of his culture material.

To control this factor two mice were placed in a dialysis jar connected with a tank of electrolytically prepared oxygen. The oxygen from the cylinder was led through glass tubes and bubbled successively through three wash bottles containing respectively 2M H_2SO_4, 2M NaOH and an approximately 10 per cent solution of K_2MnO_4. 4 days after the flow of oxygen was begun both mice were gasping for breath and incoordinated in their motions. The following day one of

Protocol.

Air Reduction Oxygen.

Nov. 24, '25. Two mice put in dialyzing jar. One stained with safranine, the other unmarked. 3.50 p.m. O_2 flow started at 1 liter per min.

Nov. 25, '25, 2.20 p.m. Both mice appear normal. O_2 concentration 96 per cent. CO_2 concentration 0.27 per cent.

Nov. 27, '25, 11.50 a.m. Mice appear drowsy and inactive. O_2 concentration 99 per cent. CO_2 concentration 0.41 per cent.

Nov. 28, '25, 8.40 p.m. Mice alive but very dyspneic.

Nov. 29, '25, 10.30 a.m. Safranine-marked mouse is dead. Second mouse living but very dyspneic. Moves about actively, however. Coat is ruffled. O_2 concentration 97 per cent. CO_2 concentration 0.11 per cent.

Nov. 30, '25. The surviving mouse breathing very rapidly, but not gasping. O_2 concentration 98 per cent.

Dec. 2, '25. Died at 11 a.m. Autopsy shows usual picture of red liver-like appearance of lungs. The removed lungs sink when placed in distilled water.

Electrolytic Oxygen.

Nov. 24, '25. Two mice put in dialyzing jar. One stained with safranine, the other unmarked. 3.50 p.m. O_2 flow started at 1 liter per min.

Nov. 25, '25, 12.30 p.m. Mice lively. Appear normal. O_2 concentration 96 per cent. CO_2 concentration 0.55 per cent.

Nov. 27, '25, 11.30 a.m. Mice appear normal, but drowsy. O_2 concentration 98 per cent. CO_2 concentration 0.50 per cent.

Nov. 28, '25, 8.40 p.m. Both mice very dyspneic.

Nov. 29, '25, 10.30 a.m. Severe dyspnea. 4.30 p.m. Unmarked mouse gasping. Lower jaw drops with each inspiration. Other mouse looks sick but is not gasping. Both move about when jar is shaken. Coats are ruffled. O_2 concentration 96 per cent. CO_2 concentration 0.18 per cent.

Nov. 30, '25. Both mice gasping. Noses and tails dusky bluish. Have eaten very little.

Dec. 1, '25. Both mice found dead. Lungs are deep purple-red the color of liver. Sink in water.

them was dead and the other, when moribund, was etherized. The lungs of both showed the usual changes.

In another experiment oxygen was bubbled through olive oil for the purpose of removing traces of ozone, even though its presence could not be shown by the starch-iodine test on the gas as it emerged from the cylinder. Mice exposed to this oxygen died of the same symptoms and showed the same autopsy findings as all the others described above.

DISCUSSION.

The phenomena described in this paper seem to have general application. The mammalian organism can survive exposure to oxygen concentrations varying roughly from 6 per cent to 60 per cent of an atmosphere. Beyond these limits it rapidly deteriorates, and at each extreme, death results from oxygen deprivation. At the lower limit oxygen want results from the diminished alveolar oxygen tension. At the upper limit, although alveolar oxygen tension is far above normal, the diffusion membrane of the lung is so damaged that in spite of the increased head of pressure in the alveoli, the arterial blood remains unsaturated, and the animal dies from anoxemia. This process possibly is of a "protective reaction" nature on the part of the organism, but going too far culminates in death. The organism, in its unsuccessful effort to achieve a new equilibrium, is kept alive by the very environmental condition which ultimately destroys it.

SUMMARY AND CONCLUSIONS.

1. Oxygen in concentrations of over 70 per cent of an atmosphere is poisonous to dogs, rabbits, guinea pigs and mice.

2. The poisonous effects manifest themselves in drowsiness, anorexia, loss of weight, increasing dyspnea, cyanosis and death from oxygen want.

3. The cause of oxygen want is a destructive lesion of the lungs.

4. The lesion may be characterized grossly as an hemorrhagic edema. Microscopically there is to be seen in varying degrees of intensity (a) capillary engorgement with hemorrhage, (b) the presence of interstitial and intraalveolar serum, (c) hypertrophy and desquama-

tion of alveolar cells, (d) interstitial and alveolar infiltration of mononuclear cells.

5. The type of tissue reaction is not characteristic of an infectious process and no organisms have been recovered at autopsy from the heart's blood or from lung puncture.

6. The poisonous effects of inhalations of oxygen-rich mixtures do not appear to be related to impurities in the oxygen, nor are they related to faulty ventilation, excessive moisture or increased carbon dioxide in the atmosphere of the chambers in which the experimental animals were confined.

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EXPLANATION OF PLATES.

PLATE 27.

FIGS. 1 and 2. Photomicrographs of sections of right lower lobe of lung of Dog 1. Magnification × 130. The pictures show the intraalveolar transudation of serum and red blood corpuscles.

FIG. 3. Section of right lower lobe of Dog 1. Magnification × 500. The picture shows desquamated alveolar epithelial cells.

PLATE 28.

FIG. 4. Section of lower lobe of right lung of Dog 3, showing engorged and tortuous alveolar capillaries. Magnification × 130. This probably represents the initial hyperemic stage before hemorrhage and edema have occurred.

FIG. 5. Section of lung of Rabbit 1. Magnification × 130. The picture shows transudation of serum and cells as well as cellular exudate.

FIG. 6. Section of lung of Rabbit 3. Magnification × 500. The picture shows desquamation of alveolar epithelial cells as well as an exudate consisting chiefly of mononuclear and a few eosinophilic cells.
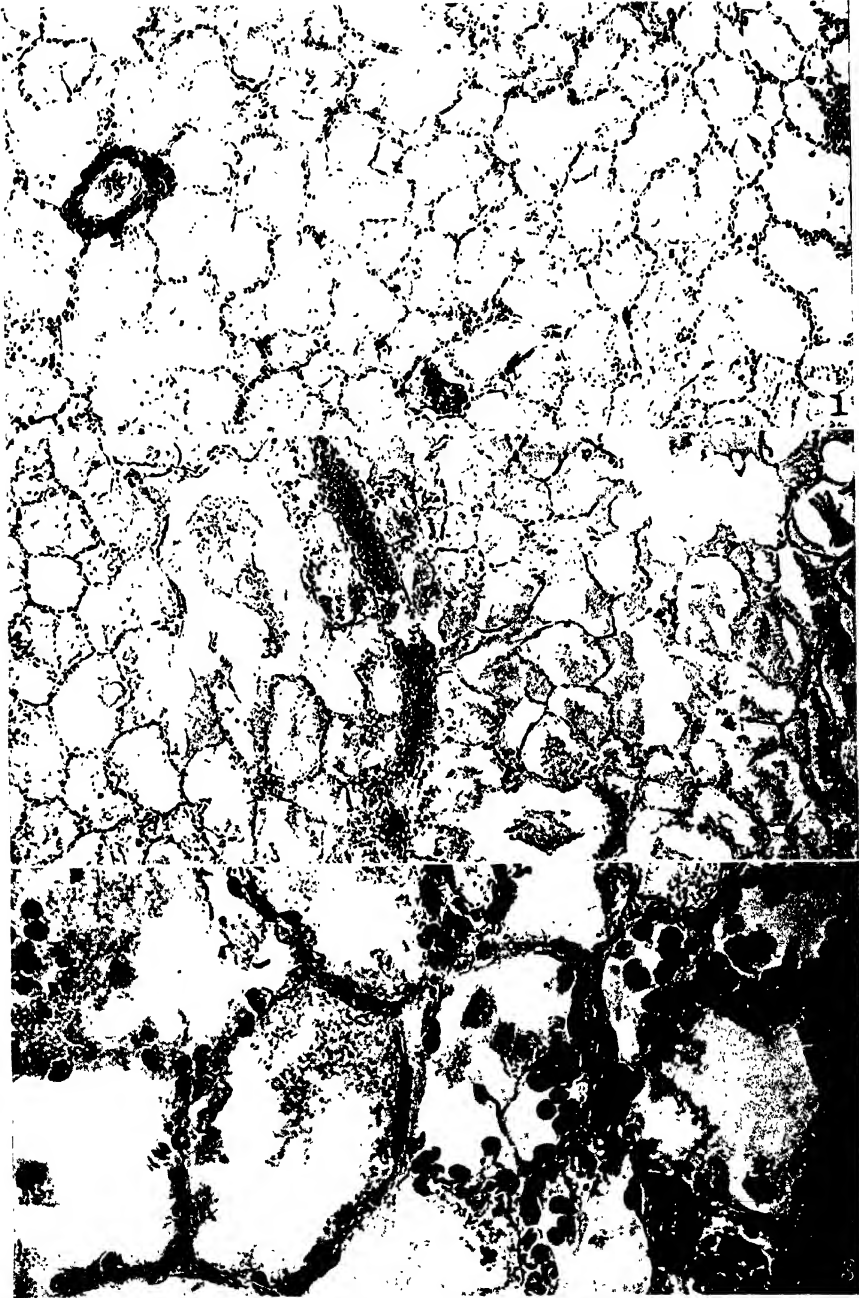
(Binger, Faulkner, and Moore: Oxygen poisoning in mammals.)
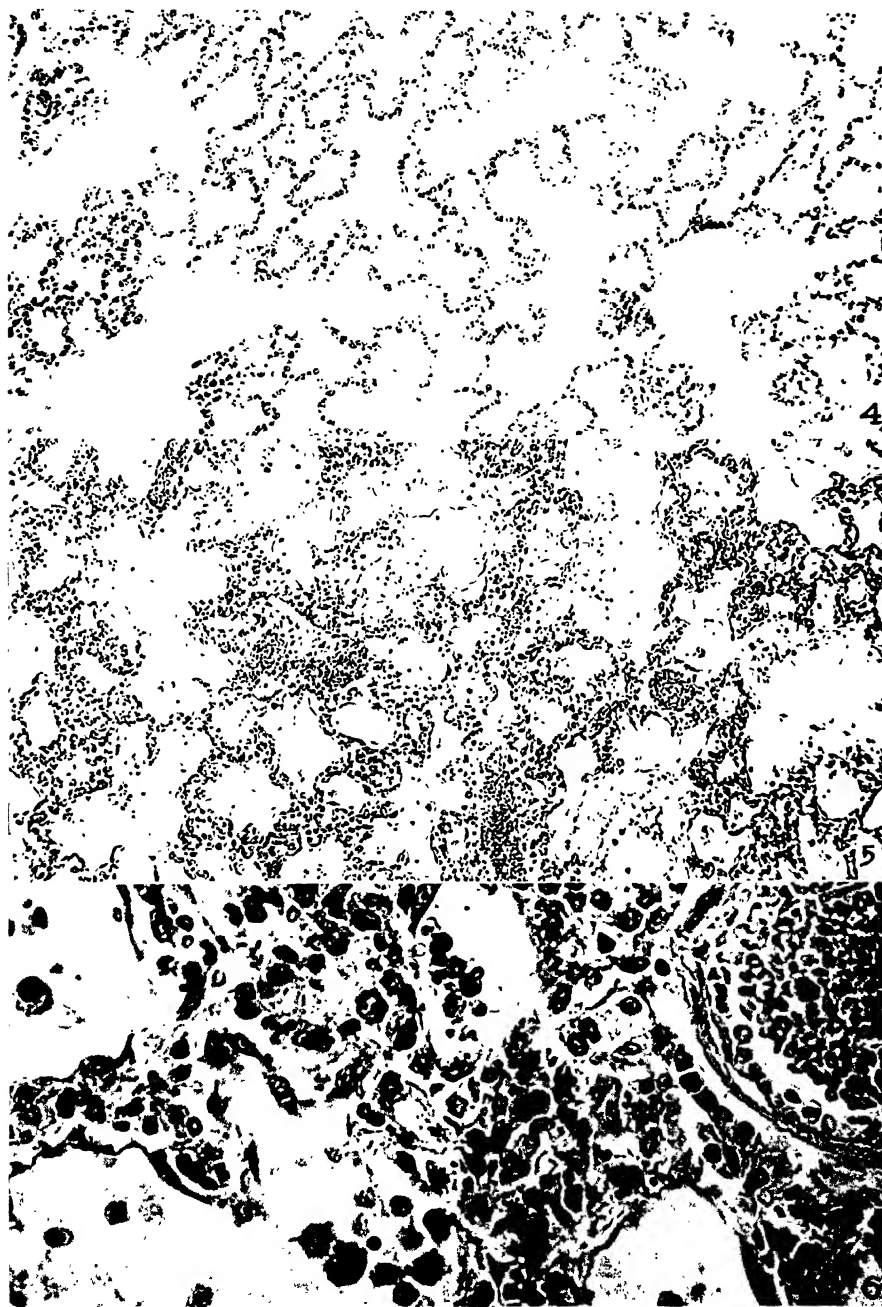
(Binger, Faulkner, and Moore: Oxygen poisoning in mammals.)

OXYGEN POISONING IN COLD BLOODED ANIMALS.

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PLATE 29.

INTRODUCTION.

In another paper (1) the effect of breathing atmospheres rich in oxygen on a variety of mammals was reported, and the nature of the injury to the lungs and the cause of death was discussed. It was pointed out that the pulmonary lesion was characterized by capillary dilatation and hemorrhagic edema. The work here to be described was undertaken not simply for the purpose of extending the study to other species but with the hope of observing the effect of high concentrations of oxygen on a capillary system more accessible to inspection than that in the lungs. For this purpose we utilized the web of frogs exposed to high concentrations of oxygen (90 to 99 per cent) over long periods. The web capillaries were examined periodically during life and after death sections were made of the skin, as well as of the lungs, heart, liver and kidney. No changes were observed in the capillaries or in the pigment cells as a result of oxygen administration. Two frogs grew definitely darker in color after 7 and 10 weeks exposure, respectively, but we attach no special significance to this, because color changes in the frog's skin are readily produced by a variety of causes difficult to control (2). In this instance inanition may have been a cause, since the frogs were not fed during the course of the experiment.

Krogh (3) has shown that the cutaneous respiratory exchange of the frog differs from its pulmonary respiration in that carbon dioxide is eliminated chiefly through the skin, while oxygen is absorbed chiefly by the lungs. Enough oxygen may be absorbed through the skin, however, to keep a frog alive for several weeks at 7-8°C. when the pulmonary ventilation is entirely cut off (4). This may be

accomplished by applying a gag to the frog in such a manner that it is unable to close its mouth, thus inhibiting the normal swallowing movements, whereby this animal forces air into its lungs (5). By placing gagged frogs in jars containing 90 to 95 per cent oxygen it was hoped to bring about changes in the skin capillaries, since the oxygen absorption would, under these circumstances, be chiefly through the skin and pharyngeal mucous membrane. None of the frogs showed any changes, however, in the skin capillaries.

These experiments, though negative with respect to the original subject of enquiry, brought out the fact that frogs could survive, apparently unharmed, in concentrations of oxygen poisonous to mammals. This observation seemed of sufficient interest to merit further study. Did it signify that cold blooded animals are resistant to the poisonous action of high concentrations of oxygen? And is the difference in their behavior from the behavior of mammals solely dependent on their differences in body temperature?

EXPERIMENTAL.

Experiments on Frogs.—Two frogs placed in a shallow dish containing a little water were continuously exposed under a bell jar to an atmosphere with an oxygen concentration averaging from 80 to 90 per cent. After 21 days exposure they were removed from the jar. Throughout the experiment and at the time of removal from the oxygen they remained apparently normal in appearance and behavior. The frogs were killed and autopsied. No gross changes in the lungs or other viscera were to be seen. Two other frogs were kept continuously in 90 to 98 per cent oxygen for respective periods of 49 and 68 days without apparent damage to their well being. At the close of the experiment they were found to be more deeply pigmented than they had been at the start, forming a marked contrast in color to two control frogs. No other changes in them were to be seen. Mammals exposed to similar environmental conditions would probably have died within the 1st week. To discover whether this difference in behavior was due to differences in body temperature an effort was made to keep frogs alive at incubator temperature (37.5°C.), but the frogs were unable to withstand this and died within 24 hours. For this reason we decided to make experiments on turtles whose life habits frequently expose them to high temperatures.

Experiments on Turtles.—Is the turtle also resistant to high concentrations of oxygen?

Protocol. Experiment 1.—A common adult pond turtle (*Chelopus guttatus*) was placed in a shallow dish containing a little water. The dish was covered with a

bell jar through which a stream of oxygen was allowed to run at the rate of 1 liter per minute. After 23 days exposure to an atmosphere containing an oxygen concentration for the most part above 90 per cent the turtle was removed in an apparently normal state. At autopsy the lungs were found to be normal in both gross and microscopic appearance (see Fig. 1).

Experiment 2.—The above experiment was repeated on another adult of the same species. After 10 days in an atmosphere of 90 per cent oxygen the turtle was removed from the bell jar in an apparently normal state.

Experiment 3.—Five young green turtles (*Chrysemes elegans*) lived for 3 weeks in 97 to 99 per cent oxygen at room temperature (23–26°C.) without appearing in any way affected by the unusual environment. A sixth member of this group was found dead after 17 days exposure, but there was no respiratory distress observed, and no definite evidence of pulmonary pathology was seen. These experiments indicate that the turtle, like the frog, can survive, unharmed, in concentrations of oxygen fatal to mammals.

Effect of Mammalian Temperature on Turtles.

Two adult specimens of *Chelopus guttatus* survived for 36 days in an incubator at 37.5°C., showing at no time loss of appetite or other signs of ill health.

An adult *Chrysemes elegans* spent 34 days, and six young individuals of the same species spent 21 days in the incubator. At the end of this time they were all lively and eating well.

From these observations it can be concluded that turtles are unharmed by prolonged exposure to room air warmed to 37.5°C.

Effect of Combination of High Concentrations of Oxygen and Mammalian Temperature on Turtles.

To find out whether the difference in the reaction of turtles exposed to high concentrations of oxygen from that of mammals was dependent upon their differences in body temperature, we exposed turtles to 90 per cent oxygen warmed to 37.5°C. Individuals of various ages and species were confined in an ordinary bacteriological incubator in which the oxygen concentration was kept above 90 per cent by blowing a stream of oxygen through it. It was soon learned that under these conditions turtles behaved like mammals in their susceptibility to oxygen, exhibiting like abnormalities in function and structure. Since young turtles behaved somewhat differently from adults the experimental results will be classed accordingly.

Experiments on Adult Turtles.

Protocol. Experiment 4.—May 22, 1926. An adult male pond turtle (*Chelopus guttatus*), kept in a shallow dish containing a little water, was put into an ordinary bacteriological incubator. This individual had previously survived, without ill effect, a 36 day exposure to room air warmed to 37.5°C. The incubator was provided with a pan of soda-lime for absorption of carbon dioxide, and a constant stream of oxygen was now run through the incubator at the rate of 1 liter per minute. Analysis at 11 p.m. showed the oxygen percentage to be 94 and the temperature 37.5°C.

May 23, 11 a.m. Oxygen concentration in the incubator 91 per cent.

May 24, 9.45 a.m. Oxygen concentration in the incubator 94 per cent.
CO_2 concentration in the incubator 0.02 per cent.

At about 11 a.m. it was noticed that the turtle was opening its mouth to breathe. It stretched its neck, opened its mouth and extended its fore legs at each inspiration. These gasping breaths increased in frequency during the afternoon.

May 25. At 10 a.m. the turtle was found dead. Autopsy showed the lungs to be opaque and blood-red instead of being of the normal pale gray transparency. Microscopic examination showed marked engorgement of the pulmonary capillaries.

Experiment 5.—May 5, 1926. Another adult turtle of the same species (*Chelopus guttatus*) was placed in an incubator containing 90 per cent oxygen, as in the experiment just reported.

May 6, 9.10 a.m. O_2 concentration 93 per cent.

7.45 p.m. O_2 concentration 94 per cent. Temperature in incubator 37.5°C. Turtle rather inactive and refuses to eat an earthworm which is offered to it.

May 7, 12.30 p.m. O_2 concentration 95 per cent. Temperature 37°C.

May 8, 11 a.m. O_2 concentration 97 per cent. Temperature 38°C.

May 9, 12.30 p.m. O_2 concentration 95 per cent. Temperature 38.5°C.

May 10, 10 a.m. O_2 concentration 95 per cent. Temperature 38.5°C. The turtle shows a peculiar form of breathing today. It stretches its neck, then opens its mouth for several seconds and swells out its throat. It then shuts its mouth, retracting and deflating its neck, apparently forcing air into the lungs. This process is repeated about every 30 seconds.

May 11, 10.30 a.m. O_2 concentration 95 per cent. Temperature 38°C. Turtle still shows same type of breathing at a somewhat more rapid rate than yesterday. Accompanying each inspiratory gasp it extends its fore legs, as if to aid respiration.

May 12, 9.40 a.m. O_2 concentration 94.5 per cent. Temperature 37°C. Same type of breathing persists.

May 13, 9.30 a.m. Oxygen cylinder emptied during night. O_2 concentration fell to 42 per cent. Gasping for breath.

10 a.m. O_2 concentration 97 per cent.

1.15 p.m. Turtle apparently lifeless—lying with its head under water. Reflexes were still present, however. The plastron was removed and the heart was

found to be still beating. All the organs except the lungs were removed. These were left *in situ* and fixed in 10 per cent formalin. The lungs were definitely redder than normal. Microscopic examination showed a very marked vascular congestion with great dilatation of capillaries. An illustration of this may be seen in Fig. 2, which should be compared with Fig. 1, a section made from the normal turtle lung.

Experiments similar to the two just described were repeated on another individual of the same species, on two adult Cumberland terrapins (*Chrysemes elegans*) and on one box turtle (*Cistudo carolina*) approximately 15 years old. All of them ended fatally. The duration of life and the degree of pulmonary congestion found at autopsy varied in the different individuals.

These experiments show that high concentrations of oxygen, when combined with incubator temperature (37-38°C.), are fatal to adult turtles of several different species, producing changes analogous to those found in the mammal, namely: progressive dyspnea, culminating in death, with hemorrhagic changes in the lungs. Neither high concentrations of oxygen, nor warm room air alone brought about these changes.

Experiments on Young Turtles (Chrysemes elegans).

It has already been stated that young individuals of the species *Chrysemes elegans* survived unharmed exposure to 97 to 99 per cent oxygen for a prolonged period. They were found to be equally resistant to exposure to warm air, behaving in these respects just like the adults.

On exposing them to oxygen warmed to 37.5°C., it was observed that they survived longer than the adult specimens did. Eventually, however, they succumbed, showing similar hemorrhagic extravasations into the lungs.

Experiment 6.—Five young turtles (*Chrysemes elegans*), whose shells measured about 3.5 cm. in diameter, were exposed in an incubator at 37.5°C. to an oxygen concentration varying from 94 to 98 per cent of an atmosphere. After 21 days exposure, the first one died. After 24 days, a second died. After 27 days, two of the survivors showed curious wing-shaped dark areas on their backs, found at autopsy to be due to seepage of extravasated blood from the lungs. These two died respectively on the 32nd and 41st day after exposure. The lungs at autopsy were found to be very red, with extravasation of blood into the extrapulmonary tissue.

The last turtle died on the 50th day after exposure to warm oxygen. At autopsy its lungs were found to be red and edematous, with blood stains through the plastron and carapace.

DISCUSSION.

An adequate explanation for the facts presented in this paper is not at hand. It is clear that increasing the temperature does not increase the oxygen concentration in the body. The implication would seem to be that a reaction occurs between oxygen and pulmonary tissue whose temperature coefficient is such that it progresses at the temperature of the mammalian body but not at ordinary room temperature. Whether a chemical substance is produced which acts as an irritant to the lungs cannot be stated, or whether the increased temperature acts by raising the metabolic rate, which is known to enhance the toxic effect of certain substances.

SUMMARY AND CONCLUSIONS.

1. Exposure of frogs to atmospheres containing approximately 95 per cent of oxygen is without apparent effect on their state of well being, and produces no noticeable changes in the appearance of their web capillaries.

2. Turtles exposed to similar atmospheres are also apparently unaffected unless the oxygen be warmed to mammalian temperature.

3. At this temperature (37.5°C.) the turtles behave like mammals, showing loss of appetite, shortness of breath, death and, at autopsy, hemorrhagic extravasations in the lungs.

4. Young turtles are more resistant (or adaptable) to this change in environment than mature ones.

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EXPLANATION OF PLATE 29.

FIG. 1.   Section of normal turtle lung showing thin walled respiratory membrane.   Magnification × 240.

FIG. 2.   Section of lung taken from turtle killed by exposure to warm oxygen. Magnification × 240.   The photograph shows a characteristic hemorrhagic area with extravasated nucleated red blood corpuscles.

(Faulkner and Binger: Oxygen poisoning in cold blooded animals.)

SPONTANEOUS INFECTIONS OF GUINEA-PIGS.

Pneumococcus, Friedländer Bacillus and Pseudotuberculosis (Eberthella caviae).

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During the course of the study of tuberculosis in small laboratory animals, one is repeatedly struck by the high percentage of common nontuberculous infections which occur in such animals. In the usual prolonged experiments on tuberculosis in guinea-pigs, for example, death from intercurrent disease is very frequent. As these animals are chiefly used in the study of this disease, deaths from such intercurrent diseases may prevent one drawing any definite conclusions, or, indeed, the experiment may be completely spoiled. For this reason Dr. Petroff suggested that it would be advantageous to study such infections since no detailed studies have been made of these common infections in guinea-pigs comparable with those of Webster[1] on the respiratory infections of rabbits (B. lepisepticum), although the reports by Theobald Smith[2] and Holman[3] are extensive.

The observations here reported were made on a colony of about 400 guinea-pigs at Trudeau Sanatorium from February to June, 1926. Half of these ("old stock") had been bred on the premises, while the remainder ("new stock") were obtained from dealers in February. The epidemic was noticed in the first week of February when a larger number than customary of the animals began to die and many were observed to have snuffles and otitis. During the epizootic 114 animals died: 56 of these were infected with the Friedländer bacillus, 36 with the pneumococcus, 5 with B. bronchisepticus, 3 with strepto-

[1] J. Exper. Med., 1926, 43, p. 573.
[2] J. Med. Research, 1913, 29, p. 291.
[3] Ibid., 1916, 35, p. 151.

coccus, 3 with organisms of the Eberthella group and in the remainder the cause of death was undetermined.

Technic.—Necropsies were performed as soon after death as possible. Culture of the right heart's blood was made on pneumococcus broth. Material from all foci of infection was smeared and cultured. The smears were stained by Gram's and Hiss' capsule methods respectively. Cultures were made on hemolyzed sheep's blood agar plates and occasionally on rabbit's blood agar plates. Sections of lung and other tissues showing lesions were fixed in Zenker's and examined histologically.

TABLE 1.

To Show Relative Mortality Incidence of Various Infections.

Organism.	Deaths.	
	Number.	%
B. Friedländer..	56	49.2
Pneumococcus..	36	31.6
Pseudotuberculosis (Eberthella)*.........................	3	2.6
Bronchisepticus...	5	4.4
Pneumonia of undetermined etiology.....................	2	1.7
Streptococcus..	3	2.6
Paratyphosus..	1	0.9
Undetermined..	8	7.0
	114	

* There were 11 other cases of this infection in which, however, the cause of death was attributed rather to the coexisting acute pneumococcus of B. Friedländer infection, with which they are therefore included.

Pneumococcus Infections.

There were 36 cases in which the pneumococcus was the sole or chief organisms isolated from the pathologic lesions. Thirteen of these were males and 23 females, of which 14 were breeders. This much greater percentage of deaths in females, and especially among the breeders (which numbered only some 60 or 70), cannot be explained on the basis of a greater number of female than male animals in the stock used, as this was approximately the same.

Selter[4] reported a small epidemic in rabbits and guinea-pigs in two consecutive winters. He quotes similar reports by Weber and Stephansky. In 1919 Theobald Smith[2] described thirty-six cases of pneumonia in guinea-pigs. Seven of these were associated with the pneumococcus alone, while in four others it was isolated along with B. bronchisepticus. He also found pneumococcus infections in two other stocks and concludes that this organism must be regarded as a widely disseminated pathogenic organism among guinea-pigs. He quotes Binaghi, Ungermann, Christiansen and Richters as describing epizootics or isolating Gram-positive diplococci from guinea-pigs. Stillman,[4a] at The Rockefeller Institute, has had four or five guinea-pig strains sent to him from outside sources, all of which were type 2 C,[4a] while Gardner, at the Saranac Lake Laboratory, frequently finds pneumococcus infections complicating his dust experiments. His strains are group 4. Holman[3] encountered the organism only three times. He quotes epidemics reported by Tartakowsky, Wittneben, Solomon and Kaspar and Kern, while Boni, like Selter, found these organisms in normal guinea-pigs.

The Pneumococcus.—The organisms were all isolated at necropsy. They were from various sources: the heart blood, lung, pleura, middle ear, frontal sinus, pericardium, peritoneum and skin. They are gram-positive, lancet-shaped, capsulated, bile soluble and ferment inulin. The final pH when grown on 7.8 broth is 6.6 to 6.4. They are not agglutinated with the standard type 1, 2 and 3 serums. A rabbit antiserum prepared from strain 68 agglutinated the homologous organisms in a dilution of 1:160 and also 6 other strains which were tested in 1:40 dilution. The virulence of the organisms is reltively low. Mice are killed regularly on intraperitoneal injection of the 1:100 dilution of an 18 hour broth culture. The dilutions above this are uncertain, the 1:1000 sometimes killing in a week. Small guinea-pigs sometimes succumb within a week to the 1:10 dilution, and $\frac{1}{4}$ cc. or over, intraperitoneally, kill regularly.

Relationship of the Pneumococcus to the Human Type.—Theobald Smith suggested that it would be interesting to test the relationship of the guinea-pig pneumococcus to the human type. We have attempted to do this in two ways; by preparing the protein fraction of the guinea-pig pneumococcus and testing it against a pneumococcus protein antiserum obtained from Avery at The Rockefeller Institute; and by agglutination tests using human group 4 strains

[4] Ztschr. f. Hyg. u. Infectionskrankh., 1906, 54, p. 347.
[4a] Personal communications.

and an antiserum prepared from a guinea-pig strain. Two protein solutions were prepared, one by breaking up the organisms in bile and the other by freezing and thawing. Both these preparations gave precipitin reactions against the pneumococcus protein antiserum. On the other hand, three human group 4 virulent strains obtained from The Rockefeller Institute tested against the guinea-pig pneumococcus antiserum failed to agglutinate.

Necropsy Findings.—The pneumococcus was isolated from the lesions in pure culture or was the predominant organism.

Septicemia occurred in thirty cases of 83.3%. In only one case was there no focus of infection found in the body and this was in a postpartum animal.

Pneumonia was present in eighteen, or 50% of all cases. Acute lobar pneumonia occurred in only one case, the whole right lung being involved. B. bronchisepticus was also isolated in this case. The remainder were all chronic processes. A characteristic lesion occurred in eight instances; this consists of multiple, greyish yellow, necrotic foci of varying size scattered over the surface of a dark purplish brown lung. All lobes, including the caudal, may be affected. Microscopically, these wedge-shaped areas resemble infarcts, have irregular edges and the base of the triangle is a thickened pleura. They are essentially necrotic tissue surrounded by a zone of polymorphonuclear leukocytes and fibrous tissue of greater or lesser thickness. In the remaining nine cases the chronic form of consolidation was not typical, but was merely a patch·of dark purple, leathery tissue situated at the tip of one or more lobes and usually small and multiple. Microscopically, congestion, atelectasis, irregular fibrosis and patchy pneumonia were seen. B. bronchisepticus was recovered from the lung along with pneumococcus in only three instances including the lobar lesion described above.

Exudative pleurisy occurred twenty-one times, or in 58% of all cases. It was coexistent with pneumonia in ten cases. A hemorrhagic fluid is characteristic and occurred in thirteen instances. One or both pleural cavities contain dark, bloody fluid and clots of blood enclosed in thin walled fibrous sacs. The exudate was serous in six cases and fibrinopurulent in one. Fibrous pleuritis occurred in three other instances.

Acute peritonitis was present in fourteen instances, or 39%. The amount of fluid varied from an unmeasurably small amount to 12 cc. The character of the fluid was serofibrinous or seropurulent in eight, hemorrhagic in three and fibrinopurulent in three. It occurred in both males and females and existed with or without pleurisy and pneumonia.

Pericarditis occurred ten times, or 28%. The exudate was hemorrhagic in five, serofibrinous in two, fibrinous in two (typical bread and butter heart) and purulent in one.

Otitis media occurred in eleven cases, or 30%. Both middle ears were involved in five cases and the right alone in six. The character of the exudate was

serous in six instances, yellow pus in four, and inspissated and dry in one. Not infrequently the lining of the cavity was definitely red and congested. The petrous bone was thickened in three cases. Otitis associated with B. Friedländer occurred in five other cases.

The frontal sinuses were deeply congested, covered with exudate from which pneumococci were recovered in smear and culture in four cases. In ten other instances there was an associated B. Friedländer.

Cellulitis—extensive inflammation of the skin of the abdomen, with reddening, thickening and edema, was present twice.

The spleen in our series excluding the seven cases with complications, was definitely enlarged in fifteen, 51.7%, of uncomplicated cases. The enlargement varied from slight increase to an organ estimated at six to eight times larger than usual. Many authors pay particular attention to this finding.

Two cases were complicated by generalized tuberculosis and five with multiple abscesses in the mesentery, liver and spleen, due to an organism of the Eberthella group described below. B. Friedländer sinusitis and otitis coexisted in five instances and sinusitis in ten others.

It will be seen from the above that hemorrhagic serous effusions are quite common in pneumococcus infections in guinea-pigs, especially hemorrhagic pleurisy. There is also a type of chronic consolidation of the lung which seems characteristic.

Analysis of the animals shows that only 5 belonged to the new stock, the remainder being all old stock. The infection occurred simultaneously throughout all the stock in different rooms and different cages. The epidemic ceased early in June. Whether this was due to the addition of grass to the food, to warmer weather, to the possibility that the susceptible animals had all died out, to a change in the character of the organism or to vaccination of the younger stock and breeders, cannot be settled now.

Friedländer Bacillus Infections.

There were 56 deaths attributed to this organism. Twenty-seven of the affected animals were males and 29 were females, including 11 breeders.

Direct allusions in the literature to infections of animals with the capsulatus group are meager. Friedländer[5] himself sprayed mice with human strains and produced pneumonia. R. Pfeiffer[6] in 1889 described the first case in guinea-pigs

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[5] Friedländer: Virchow's Arch., 1882, 87, p. 319.

[6] Pfeiffer, R.: Ztschr. f. Hyg., 1889, 6, p. 145.

(B. Capsulatus Pfeiffer). Klein[7] working with B. pneumosepticus described an organism which is possibly in this group. Weaver[8] in 1898 found probably a B. mucosus capsulatus, and Smith[9] in 1894 isolated B. lactis-aerogenes once. Perkins[10] in 1901 described an epidemic in guinea-pigs associated with B. mucosus capsulatus in which the characteristic feature was a gaseous emphysema of the liver and spleen. Holman[3] in 1916 found B. lactis-aerogenes in forty-eight animals and B. acidilactici in two. Endometritis occurred most frequently. Avery and Heidelberger[11] used a guinea-pig strain in their work on the soluble specific substance of B. Friedländer and Julianelle[12] used such a strain in typing organisms of this group. Gardner at the Saranac Lake Laboratory, Petroff at the Trudeau Laboratory and Stillman at The Rockefeller Institute are familiar with its occurrence in guinea-pigs. Stillman and Branch[13] used a guinea-pig strain in their work on experimental B. Friedländer pneumonia in mice. Jensen[14] found a capsulated organism in the normal intestine (B. centropunctatus). Chrom[15] thinks guinea-pigs particularly susceptible to B. Friedländer. Sisson and Walker[16] were able to infect cats experimentally. Chester[17] studied a case of pneumonia in a calf associated with B. mucosus capsulatus.

The Organism.—The strains were obtained at necropsy from many sources, i.e., the heart blood, lung, pleura, pericardium, peritoneum, middle ear or sinus. The organisms are large, gram-negative, plump rods, which vary in length and have a definite capsule. They are nonmotile, grow readily on broth, forming a heavy pellicle and diffuse turbidity. The agar colonies are highly refractile, sticky and are the color of pale gelatin. All the strains are gas formers, dextrose, saccharose, mannitol, levulose, maltose, salicin, galactose, arabinose and xylose being fermented, while lactose, dulcitol and adonitol are not fermented. Gelatin is not liquefied nor is indol formed; nitrates are reduced and H_2S is formed.

Serologic Reactions.—The organisms agglutinate rapidly in type 2 pneumococcus serum and also in type B Friedländer bacillus serum obtained from Julianelle at The Rockefeller Institute, so that they undoubtedly fall into type B of the Friedländer organisms.

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[7] Centralbl. f. Bakteriol., 1, O., 1889, 5, p. 625; ibid., 1891, 10, pp. 619, 841; ibid., 26, p. 260; Twenty-Second Report Local Government Board, 1892–3, 367; Centralbl. f. Bakteriol., 1, O., 1905, 38, p. 392. (Quoted by Holman.)

[8] Trans. Chicago Path. Sc., 1897–99, 3, p. 228. (Quoted by Holman.)

[9] Centralbl. f. Bakteriol., 1, O., 1894, 16, p. 237; J. M. Research, 1905, 13, p. 341; ibid., 1913, 29, p. 291. (Quoted by Holman.)

[10] J. Exper. Med., 1900, 5, p. 389.

[11] J. Exper. Med., 1925, 42, p. 701.

[12] Ibid., 1926, 44, pp. 113, 683.

[13] Ibid., 1925, 41, p. 623.

[14] Manual of Bacteriology, 1909, p. 225. (Quoted by Holman.)

[15] Centralbl. f. Bakteriol., 1, O., 1911, 59, p. 103. (Quoted by Holman.)

[16] J. Exper. Med., 1915, 22, p. 747.

[17] Twelfth Annual Report of the Delaware College Agric. Exper. Sta., 1900.

Virulence: Mice, inoculated intraperitoneally, are rapidly killed with a 0.000001 dilution, the highest used. Small guinea-pigs succumb rapidly in the 1:10 dilution of an eighteen hour broth culture. Twelve guinea-pigs from an outside stock, which, by nasal swab examination, were first shown not to be Friedländer bacillus carriers, on three occasions had ⅓ cc. of an eighteen hour culture run into either nostril. Two of these animals succumbed with acute B. Friedländer pneumonia and septicemia in five to six days respectively. Animals from our own stock are refractile to inhalation and seldom become infected.

Formation of an Avirulent Strain: Julianelle[12] has obtained several avirulent Friedländer strains by growing the organisms on the antiserum, as has previously been shown with pneumococcus (Reimann[18]). We were able to obtain an avirulent strain by the method used by Webster[1] in producing avirulent strains of B. lepisepticus. This organism gave the same sugar reactions as the type strain but failed to agglutinate in type serum. The method is as follows: The pellicle from a four day growth on lactose broth was removed, diluted and plated. After forty-eight hours' growth a colony differing from the characteristic colony was removed and subcultivated on lactose broth for four days. This was again re-plated and the resulting colony was smaller than the characteristic colony, not refractile or sticky and could be pushed along the surface of the agar plate without adhering. The growth on fluid media is very flocculent and granular. This organism kills mice in 0.25 cc. doses intraperitoneally but not in higher dilutions.

Necropsy Findings.—B. Friedländer was isolated from all the lesions.

Septicemia occurred in thirty-five of the fifty-six cases or 62.5%. It was most frequently associated with local lesions in the respiratory tract and external ear, i.e., sinusitis, pneumonia and otitis. The point of entrance in five cases was apparently the skin (cellulitis in three and abscess in two).

Sinusitis was most frequent, occurring in forty-five cases, or 80%. The turbinates were congested to a varying degree and exudate was present in varying amounts in all cases. In the majority it was sticky, mucoid material or greyish mucosus. In four animals necrosis of the turbinates had taken place. Otitis was a coincident lesion thirty-one times and pneumonia twenty-four times.

The middle ears were involved in thiry-seven of the animals or 66%. This was the second most common lesion. It was bilateral in twenty-nine cases and unilateral in eight, the left ear being affected alone in five and the right alone in three. The petrous part of the temporal bone was soft and necrotic in eleven cases and definitely thickened and resistant to the scissors in eight. The exudate was usually a thick, creamy, sticky pus. In some instances the external auditory canal contained dirty dried pus. Sinusitis was present in all but five cases and septicemia in nineteen.

Extradural abscess was a complication of ten of the otitis cases in which necrosis of the petrous portion of the temporal bone had occurred. In every case the creamy pus collected above the tentorium and encroached upon the

[18] J. Exper. Med., 1925, 41 p. 587.

temporal lobe, depressing it 1 to 2 cm. It occurred on the right side in seven instances, on the left in three and was bilateral in one. Septicemia was present in only two cases.

Abscess under the scalp occurred in five of the above eight cases of extradural abscess. The pus had burrowed through the parietal bone, which was either necrotic or showed a large orifice and formed a well recognizable, soft, fluctuating mass on the head under the scalp. The right parietal bone was eroded in three cases, and the left parietal in two. In no case was septicemia present.

Pneumonia as a definite gross and microscopic consolidation of the lung, occurred in twenty-six animals or 46%. In seven animals, three of which were young, there was typical acute B. Friedländer pneumonia. The lesion was lobar in distribution and one or more lobes were affected. In the remaining nineteen cases, both histologically and in the gross, the lesions were old and chronic, usually multiple and involving the anterior or upper tip of the cephalic and ventral lobes and the medial lobes. The consolidation was usually greyish red in color and gelatinous with slight corrugation of the surface, but often it was red and beefy and once cartilaginous. Sinusitis was a coexisting lesion in all but two cases and septicemia occurred in nineteen, six of which were the acute pneumonias.

Pleuritis was present in fourteen cases or 20%, ten times with pneumonia, four of which were in the acute consolidation. It was bilateral in twelve cases and occurred on the left side alone in two. The effusion was serous in three, bloody in one, fibrinopurulent in nine and in one a localized encysted pocket of pus was found. Septicemia was present in twelve cases.

Pericarditis occurred in six instances, all with septicemia and pneumonia. The exudate in the pericardial cavity was mucoid in four, serofibrinous in one, and fibrinous and hemorrhagic in one.

Peritonitis occurred six times. In every case there was septicemia. The exudate was fibrinopurulent in three, serous in two and serofibrinous in one.

A diffuse, widespread cellulitis of the abdomen and side occurred in three cases. In all of these there were abrasions of the skin, probably bites, and septicemia was concomitant.

Skin abscess with a pocket of thick, yellow, flocculent pus was found in four cases, in two of which there was septicemia and inflammation of one or more serous cavities.

Metritis occurred in two cases. The uterus was large and red, the wall was thickened and filled with pus. The mucosa was very injected.

Complications: Generalized tuberculosis was present in one case and pseudotuberculosis in seven. In two cases of otitis the pneumococcus was isolated and in one B. bronchisepticus. B. bronchisepticus was also recovered from a pneumonic patch in one instance.

Microscopic Examination of the Lungs.—Acute Cases: These were all typical Friedländer bacillus acute pneumonias such as have been produced experimentally (Stillman[13]).

Chronic Cases: No typical histologic picture occurred. The lungs were usually

atelectatic and the alveolar walls sometimes congested. Mononuclear cells occupied the alveoli in varying degrees and more or less widespread fibrosis and fatty degeneration were to be seen. Adenomatous-like formations around the bronchi were not uncommon. Occasionally small abscesses occurred.

Table 2 shows the relative frequency of the various lesions associated with B. Friedländer and with pneumococcus. It will be noted that sinusitis and otitis are more commonly associated with B. Friedländer, whereas generalized and acute inflammations of serous cavities are more often associated with the pneumococcus.

TABLE 2.

The Relative Incidence of the Various Lesions.

	Septicemia.	Pneumonia.	Pleurisy.	Peritonitis.	Pericarditis.	Otitis.	Sinusitis.	Extradural abscess.	Cellulitis.
With Pneumococcus Infections									
Number of animals............ (Total, 36; 23 females)	30	18	21	14	10	11	4	0	2
Percentage..................	83	50	58	39	28	30	11	0	5.6
With Friedländer Bacillus Infections									
Number of animals............ (Total, 56; 29 females)	35	26	14	6	6	37	45	10	3
Percentage..................	62.5	46	20	10.7	10.7	66	80	17.8	5.3

Eberthella caviae (Pseudotuberculosis).

Fourteen animals died with lesions of pseudotuberculosis, from 12 of which an organism of the Eberthella group (Bergey's[19] classification) was isolated. Twelve were females, seven being from the breeding cages. The infection was localized to five cages in two rooms.

Many organisms have been described as associated with the lesions of pseudotuberculosis but the organism here described cannot be absolutely identified with any one of them, though it most closely resembles the organism originally described by Pfeiffer.

[19] Manual of Determinative Bacteriology, 1926.

The literature is reviewed from the point of view of etiology.

B. Paratyphosus Group: These are perhaps the organisms which produce most characteristically multiple abscesses in rodents. They include B. paratyphosus, B. enteritidis (Gaertner), B. aertrycke and B. paratyphosus A (Kirch). The earlier descriptions[20] are inadequate for actual classification of the organisms. Theobald Smith[9] in 1894 produced the first evidence and later proved the point with B. suisepticus. Durham[21] produced the lesions experimentally with B. enteritidis (Gaertner) by feeding and this was done later by Dieterlen and Kirch. Others[22] also report epizootics. Wherry[23] described B. pestis caviae and Petrie and O'Brien[24] lost all but twenty-one of 500 animals with B. aertrycke infection. Bainbridge and O'Brien[25] found abscesses in the mesenteric glands. B. caseolyticus (Lochmann[26]), a variety of B. coli thought identical with B. caseolyticus (Kovarzik[27]) and B. pseudotuberculare orchitophlogenes (Cagnetto[28]), probably also fall into this group.

Streptococci: They have been described by Chantemesse, Dor,[20] Boxmeyer,[29] and others.[30] Boxmeyer's[29] epidemic was quite an extensive one and more than 100 necropsies were done. The cervical, submental and auricular glands were affected in 90% of the cases, the others less frequently. In a few cases an organism of the hog cholera group was isolated as well as streptococci. He produced the disease experimentally by feeding the hemolytic streptococcus isolated. Pasteurella group: Byloff[31] in 1906 described a typical pseudotuberculosis in guinea-pigs. He isolated an organism which he classified in the hemorrhagic septicemia group and produced the disease experimentally (B. pestis intestinalis caviae cob.). He considered it the same as an organism previously described by

[20] Malassez and Vignal: Kolle and Wassermann, Handbuch der pathogenen Mikroorganismen, 1912, 5, p. 776; Chantemesse: Ann. de l'Inst. Pasteur, 1887, l, p. 97; Charrin and Rogers: Centralbl. f. Bakteriol., 1, O., p. 1888, 4, p. 44; Dor: Compt. rend. de l'Acad. sci., 1888, 106, p. 1027.

[21] Brit. M. J., 1898, 2, p. 600; ibid., 1899, 1, p. 1216.

[22] Dieterlen: Centralbl. f. Bakteriol., 1, Ref., 1909, 44, p. 282; MacConkey: J. Hyg., 1905, 5, p. 333; de Basi: Centralbl. f. Bakteriol., 1, Ref., 1909, 44, p. 86; Eckersdorf: ibid., 1906, 38, p. 99.

[23] J. Infect. Dis., 1908, 5, p. 519.

[24] J. Hyg., 1910, 10, p. 287.

[25] J. Path. & Bact., 1911, 16, p. 145.

[26] Centralbl. f. Bakteriol., 1, O., 1902, 31, p. 385.

[27] Ibid., 33, p. 143.

[28] Ann. de l'Inst. Pasteur, 1905, 19, p. 449.

[29] J. Infect. Dis., 1907, 4, p. 657.

[30] Eberth: Virchow's Arch., 1885, 100, p. 15; Lamar: J. Exper. Med., 1909, 11, p. 152; Horne: Centralbl. f. Bakteriol., 1, O., 1912, 66, p. 169.

[31] Centralbl. f. Bakteriol., 1, O., 1906, 41, pp. 707, 787; 42, p. 5.

Fig. 1.

A   **B**
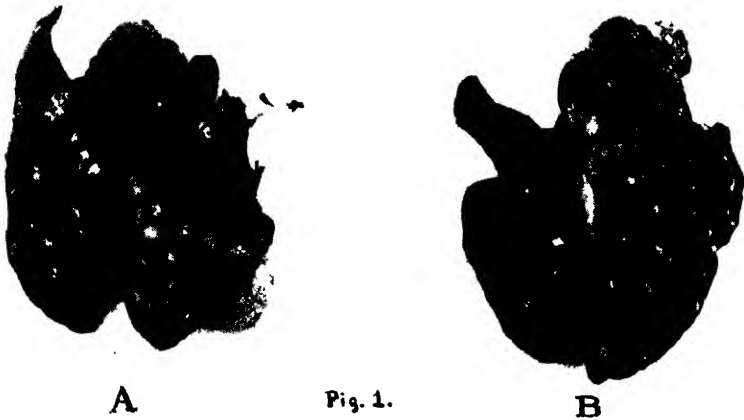
Fig. 1.- (A) Photograph of guinea-pig's lungs showing the lesions of pseudo-tuberculosis (Eberthella caviae). (B) Photograph of guinea-pig's lungs to show for comparison tuberculosis of the lung (Mycobacterium tuberculosis humanis) occurring in generalized experimental tuberculosis.
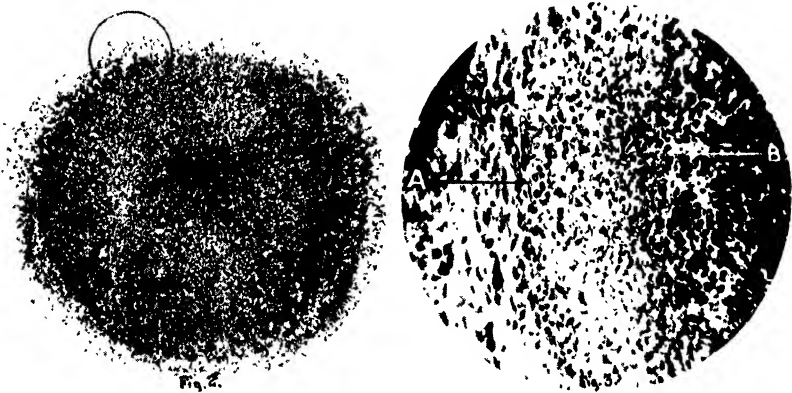
Fig. 2.—Microphotograph of a pseudotubercle in the liver of a guinea-pig. Low magnification. Circle: portion shown in fig. 3.

Fig. 3.- -Microphotograph of the periphery of the nodule seen in fig. 2. A: the interior of the nodule composed of a mass of cells. B: the fibrous wall. Higher magnification.

Zalatogoroff[32] and A. Pfeiffer[33] in spite of its motility. Dieter and Rhodes[34] have encountered lesions in guinea-pigs which resemble plaque and are due to bipolar organisms not culturally B. pestis. B. Coli: An organism of this group is described,[35] producing either pseudotuberculosis or abscess. Micrococcus tetragenous: Kasper and Kern[36] describe numerous abscesses associated with this organism mixed in two cases with a gram-negative and in one with a pneumococcus. Spore formers: Eberth and Dor recovered these from cases of pseudotuberculosis. Coccidea: Cases of coccidiosis are described.[37] Natural plague: Holman[3] points out that this occurs spontaneously in India. Tularemia: Dieter and Rhodes[34] have recently reported lesions in guinea-pigs produced by injecting material from wild oats suspected of carrying B. pestis, which resembled the lesions of experimental plague but were due to B. tularense. ·B. abortus: Schroeder in Washington showed Petroff lesions caused by this organism in guinea-pigs and he was struck with the superficial resemblance to tuberculosis. Blastomycosis: Long[37a] states that injection of this organism causes in guinea-pigs multiple abscesses not unlike tuberculosis. Rivas,[38] mentions that blastomycosis in animals may be produced by another genus of Saccharomyces, i.e., the cryptococcus. Diphtheroid group: Several authors[39] have described the occurrence of the corynebacterium rodentium in pseudotuberculosis of rodents.

Gross Pathologic Findings.—The mesenteric glands were involved in every case. The glands varied in size from an almond to a fused mass the size of a full term guinea-pig fetus. The gland at the junction of the cecum and ileum was always involved but usually not greatly enlarged. The glands were completely transformed into a mass of thick, putty-like, yellowish white pus and fibrous tissue. The capsule showed great fibrous thickening, the gland appearing pearly white. The liver and spleen were affected in ten instances, 71%. The spleen was enlarged, sometimes six to eight times the normal size. The surfaces of both organs were studded with small round white nodules. These were apt to be discrete and varied in diameter from 1 to 4 mm. The edges were well outlined from

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[32] Centralbl. f. Bakteriol., 1, O., 1904, 37, p. 654.

[33] Ueber die Bacilläre Pseudotuberkulose bei Nagatieren, 1889.

[34] J. Infect. Dis., 1926, 38, p. 541.

[35] Galli-Valerio: Centralbl. f. Bakteriol., 1, Ref., 1900, 27, p. 305; Schwartz: Ibid., 1, O., 1906, 36, p. 273; Simon: Compt. rend. Soc. de biol., 1910, 69, p. 393.

[36] Centralbl. f. Bakteriol. 1, O., 1912, 63, p. 7.

[37] Strada and Traina: Centralbl. f. Bakteriol., 1, O., 1900, 28, p. 635; Pianese: Ztschr. f. Hyg., 1901, 36, p. 350; Griffith: Report Royal Comm. Tuberculosis, 1907, 1, pt. 2.

[37a] Personal communication.

[38] Textbook on Human Parasitology, 1920, p. 594.

[39] Pfeiffer: Ueber die Bacilläre Pseudotuberkulose bei Nagatieren, 1889; Preisz: Ann. de l'Inst. Pasteur, 1894, 8, p. 231; Migula: System der Bakterien, 1900, p. 374. Cited from Bergey,[19] p. 386.

TABLE 3.

Pseudotuberculosis.

Guinea-pigs.			Organism.	Organs affected.						Complications.
No.	Sex.	Cage.		Mesenteric glands.	Liver.	Spleen.	Omentum.	Lungs.	Gut.	
1	Female	B 1	Eberthella	+	+	+	0	+	+	Friedländer's otitis and sinusitis
2	Female	B 1	Friedländer	+	0	+	+	0	0	Friedländer's septicemia
3	Female	A 3	Eberthella	+	+	+	+	0	+	Friedländer's sinusitis, pneumococcus otitis
4	Female	B 1	Friedländer	+	+	+	0	0	+	Friedländer's septicemia and pneumonia
5	Female (breeder)	A 2	Eberthella	+	+	+	0	+	+	
6	Female (breeder)	A 2	Eberthella	+	+	+	+	0	0	
7	Female (breeder)	A 3	Eberthella	+	0	0	0	0	+	
8	Female (breeder)	A 3	Eberthella	+	0	0	0	0	+	Pneumococcus septicemia
9	Male	B 8	Eberthella	+	+	+	+	+	+	Friedländer's septicemia
10	Female	B 1	Eberthella	+	0	+	0	+	0	Pneumococcus septicemia
11	Female (breeder)	A 2	Eberthella	+	+	+	0	+	0	Friedländer's otitis
12	Male	B 2	Eberthella	+	+	0	0	+	0	Pneumococcus septicemia
13	Female (breeder)	A 3	Eberthella	+	+	0	0	0	0	Pneumococcus septicemia
14	Female	B 1	Eberthella	+	+	+	+	+	+	Friedländer's septicemia
Total number (Eberthella 12) (Friedländer's 2) 14				14	10	10	5	7	8	
%				100	71	71	36	50	57	

the surrounding tissue and in some cases the abscesses were slightly but definitely raised above the surface. Each nodule was filled with pus similar to that found in the mesenteric glands. The intestine showed a few small abscesses in eight cases. These were practically always in the ascending colon, less often in the cecum and occasionally in the small gut. The lung showed numerous small scattered yellow abscesses surrounded by a narrow zone of congestion in seven cases. The omentum was involved in five cases. The organ was rolled up around the inferior surface of the stomach and showed numerous small abscesses.

Microscopic Examination.—The nodules are sharply outlined from the surrounding liver structure by a well developed layer of fibrous tissue. In the liver the contiguous layer of liver tissue shows degeneration, and a fatty metamorphosis of the whole liver is quite frequent. The interior of the nodule is composed of a mass of cells and nuclear debris. Most of the cells are mononuclears but there are polymorphonuclears, particularly around the periphery. In the lung the lesions are not as well walled off by a fibrous capsule. In the gut the abscesses are situated in the submucosa. The caseous glands are really composed of a series of small abscesses surrounded by dense fibrous tissue which fuse in the later stages. In the spleen they appear to occur chiefly in Malphigian corpuscles and the pulp is atrophic. The omental lesions are in the substance of the organ.

The Organism.—As stated above an organism of the Eberthella group was isolated in twelve of fourteen cases. In the other two cases B. Friedländer was recovered. Both were, however, complicated by B. Friedländer septicemia, so that probably technical difficulties account for the failure to isolate the Eberthella organism.

The organism as isolated from the abscesses in various organs in a plump, gram-negative, nonmotile bacillus, measuring in broth cultures 0.5 × 0.8 to 2 microns. On gelatin coccoid forms are more common, whereas on agar with 5% sodium chloride longer rods than the above are found. Involution forms occur in old cultures. No polar staining, no spores and no capsules were demonstrated. In the direct smear of the pus from one of the abscesses, it is extremely difficult to find organisms. They are few in number and in some instances we failed to find any. No acid fast organisms were ever demonstrated. They grow well aerobically. On broth a heavy sediment forms with a slight pellicle. On agar the colonies in twenty-four hours are less than 1 mm. in size and the edges are somewhat rough, the outline being irregularly octagonal. Maximum growth occurs in forty-eight hours when the individual colonies are 1.5 to 2 mm. in size. They are not strictly confluent though they may be in apposition. The colonies are translucent and bluish grey, resembling the colonies of B. bronchisepticus at first. They are convex, the center being distinctly raised but not granular. They do not adhere to the needle, nor are they very sticky. Gelatin is not liquefied and the growth here is heavy, arborescent on the surface and with fine, delicate, coral-like offshoots in the deeper parts. The general characteristics are summarized as follows:

Eberthella caviae: Biologic and Cultural Characteristics.

Gram-negative
No polar staining
Size: 0.5 by 0.8 to 2.0 microns
Nonmotile
No capsules, nor spores
Broth: sediment and pellicle
Agar: translucent colonies 2 mm.
Gelatin: aborescent, no liquefaction

Voges-Proskauer reaction negative
Indol not formed
Nitrates reduced
Hydrogen sulphide
Litmus milk, alkaline

Carbohydrate Reactions.

Acid produced in
    Dextrose, galactose, levulose, maltose, mannose, xylose, mannitol, dulcitol, salicin
Agglutination negative with antiserums for B. typhosus, B. paratyphosus A and B, B. dysenteriae (Flexner and Hiss)

No fermentation in
    Saccharose, lactose, arabinose, adonitol, dextrin

Virulent for mice and guinea-pigs

These findings were corroborated by L. T. Webster, by Holman and by Bergey who says that in the absence of polar staining the organism undoubtedly falls into the Eberthella group. He adds the following additional characteristics: Russell's double sugar agar shows acid base but no gas. There is no action on lead acetate in this medium. Tests with immune serums showed no agglutination with antityphoid, antiparatyphoid A and B, antidysentery or antiparadysentery (Flexner and Hiss) serums. Holman thinks the organism comes closer to B. pseudotuberculosis rodentium (Pfeiffer) than to any other.

In view of the fact that this organism shows carbohydrate fermentations like some strains of Friedländer bacillus and as infection with B. Friedländer has been common in our stock, it seemed necessary to exclude the possibility of its being an avirulent B. Friedländer organism. This we think we have done by agglutination tests. The organisms were cross agglutinated against two antiserums obtained from The Rockefeller Institute prepared by using avirulent Friedländer organisms. The results were negative. Similarly, agglutination tests using a rabbit antiserum made from a strain of these organisms against two avirulent B. Friedländer strains (one of ours and one of Dr. Julianelle's) were negative. Further evidence against it being an avirulent B. Friedländer appears below, since, unlike B. Friedländer strains, they produce lesions in guinea-pigs.

Virulence: Mice inoculated intraperitoneally with a 24 hour broth

culture are killed with 0.25 cc. and over. Small guinea-pigs in-
oculated intraperitoneally with 0.25 cc. die in three to four days with
serous peritonitis and abscesses in the liver, spleen and omentum.
Subcutaneously death ensues after a similar dose in 10 days. The
local glands show caseation; there is marked reaction sometimes with
necrosis at the site of inoculation and abscesses in the liver and
spleen more numerous.

Experimental Infection by Mouth.—One cc. of a 24 hour broth cul-
ture was fed by a stomach tube to a full grown female. In five
weeks the animal was killed and showed no pathologic lesions.

At the same time, using the same culture and similar procedure the
tube was introduced into the posterior pharynx and some regurgitated
through the mouth. This animal died in three weeks and the necropsy
findings follow:

In the neck are four large lymph glands, measuring 1 cm. in diameter, filled
with caseous pus. The mesenteric glands are slightly enlarged but grossly normal.
The hepatic gland shows many small caseous foci. There are 3 cc. of clear
serous fluid in the abdomen. The spleen is enlarged to twice its normal size.
There are many pin-head sized abscesses and a few 3 mm. in diameter on the
surface and in the deep splenic tissue. These contain thick, putty-like, yellowish
white pus. The liver is studded with small abscesses. The omentum is rolled
up in the upper abdomen and shows many small abscesses. There are a few
abscesses in the mesocolon and on the parietal peritoneum of the lower left ab-
dominal wall. There are many small, round, yellowish foci in the lungs around
which are areas of congestion. The heart blood culture was negative. A few,
short, plump, gram-negative rods were seen in the direct smear from the abscesses
in the cervical glands, the liver and spleen. In the neck glands there were also a
few cocci. No acidfast bacilli were demonstrated. The Eberthella organism
was cultivated from the cervical glands and also gram-positive cocci.

The pus from one of the cervical lymph glands was dissolved in saline and 1 cc.
inoculated into 2 guinea-pigs, one receiving 1 cc. of the emulsion intraperitoneally
and the other 1 cc. subcutaneously. The first animal died in 6 days and showed
some free peritoneal fluid and multiple abscesses in the spleen, which was enlarged,
and in the liver and lung. Smears of the pus showed gram-negative rods and no
acidfast bacilli. The second animal which received the subcutaneous inocula-
tion died in 9 days. There was an indurated necrotic focus surrounded by an
area of congestion 1.5 cm. in diameter at the site of inoculation in the groin.
Two of the local inguinal glands measured 7 mm. in diameter and were filled
with caseous pus. The liver, spleen and lung showed multiple small abscesses.
Smears of the pus showed gram-negative rods and gram-positive cocci. Four

other animals inoculated with similar doses of two avirulent Friedländer strains remained well.

A third animal was also inoculated in the groin with caseous material from the cervical glands, the pus having been previously treated with N/1 NaOH for ½ hour in the incubator. The centrifugalized material was used for inoculation and cultured on egg medium and Petroff's medium. The cultures grew and the inoculated guinea-pigs developed enlarged glands locally palpable from the fourth day and growing larger until the animal was killed on the thirteenth day. At necropsy there were four large caseous inguinal glands in the left groin, the largest being 1.5 cm. in diameter. The left iliac gland was also greatly enlarged and caseous. The rest of the necropsy was negative.

Experimental Infection by Inhalation.—Three small male guinea-pigs were exposed in a spray chamber to a mist produced by an atomizer which contained an emulsion of an 18 hour agar growth of the organisms in saline. The first animal died in 10 days, the second in 12 and the third in 14 days. The animals appeared perfectly well until about 36 hours before death when they appeared sick. The necropsy findings in the lungs in all cases were identical.

There were loose fibrinous adhesions between the visceral and parietal pleura. The lungs showed many large greyish yellow areas scattered through all lobes. There were some 4 mm. in diameter with irregular borders and they were surrounded by areas of congestion. The bronchial lymph glands were large, congested and showed scattered caseous foci. The heart blood culture was positive (Eberthella caviae) in only one instance. In two of the animals there was no other finding but in the third, which lived longest, there were two abscesses in both the liver and spleen. The specific organism was cultured in every case from the local lesions and the smears were negative for acidfast bacilli. In the x-ray examination of one of the animals, the day before death, many shadows which suggested a bronchopneumonia were shown in the lungs.

Microscopically the lesions in the lungs of the experimentally infected animals resemble those seen in the spontaneously infected except that the lesion is rather more acute, more extensive and contains more organisms. It will thus be seen that the lesions develop much more rapidly than in the case of true tubercle.

Method of Infection in the Spontaneous Cases.—We have failed experimentally (by inoculations subcutaneously and intraperitoneally, by inhalation and by mouth) to reproduce the disease exactly as it occurred spontaneously. In view of the fact, however, that one of the animals in which a culture was introduced into the throat developed local caseous glands and multiple abscesses, we feel that in

all probability the infection naturally occurs by way of the digestive tract or in females through the uterus, the mesenteric glands being thus first involved.   The fact that all animals fed by mouth do not develop the disease might account for the absence of infection in other animals in the same cage and also for the small number of animals dying of the disease.   The disease undoubtedly spreads by the lymphatics, the lymph glands early showing greater involvement than the site of inoculation.

Identification of the Organism.—The organism described as associated with pseudotuberculosis in the present epidemic belongs to either the Eberthella or the Pasteurella groups in Bergey's classification. The latter would seem the more plausible but in the absence of polar staining and involution forms and by the relative large number of carbohydrates fermented, we feel justified in placing it, at least temporarily, in the Eberthella group, even though there is no cross agglutination with other members of this group.   The growth on gelatin is somewhat arborescent and they are hardy organisms apparently withstanding treatment with sodium hydroxide.

RESUMÉ AND DISCUSSION.

In our experience spontaneous infections are relatively common among guinea-pigs.   B. Friedländer and pneumococcus, group 4, are the organisms most frequently isolated from the lesions in our own stock at Trudeau Sanatorium Laboratory, and not B. bronchisepticus or B. paratyphosus which are found more often in some localities. Besides the reports already cited certain others[39a] also describe such infections of guinea-pigs.   The extreme chronicity of some of the lesions is of interest and importance as this chronic focus of infection may play some part in altering the resistance of the infected animal to experimental inoculation with tubercle bacilli and lead to false conclusions.   Petroff and Stewart[40] have observed that animals which show extensive tuberculosis after inoculation rarely develop other

[39a] Cotoni, Truche and Raphael: Pneumococcus and Pneumococcal Affections (Translation), 1924; Thomas: J. Infect. Dis., 1924, 35, p. 407; Howell, Katharine M., and Schultz, O. T.: J. Infect. Dis., 1922, 30, p. 516; Trawinski, A.: Centralbl. f. Bakteriol., 1, O., 1922, 88, p. 24.
[40] J. Immunol., 1926, 12, p. 97.

infections (e.g., pneumonia), and the reverse may also hold. We have followed animals with snuffles and otitis clinically and with nasal swabbing over a period of months and find that at necropsy these lesions are the cause of death. Although the majority of these animals may show signs of disease, for instance, exudate in the nares, incoordination when the otitis is unilateral or emaciation, many have been found without visible local signs of infection. This is so if the otitis is bilateral as it is most frequently. Many of the animals may also be extremely well nourished with sleek coats and persist in this state for eight months, the period over which our observations have extended. One would also stress the necessity of opening the sinuses and middle ears at necropsy in every case, as we have found a very high percentage of otitis and sinusitis in our necropsies. In about 6% of the cases the cause of death would otherwise not have been found while if blood cultures are not done routinely, this percentage would be considerably higher. Why the infections are more common in the winter and may acquire epidemic proportions we cannot say, but feel that possibly the absence of sufficient green vegetables may be partly responsible. The portal of entry in most cases appears to be by the upper respiratory tract, middle ear and uterus. That chronic pulmonary infections associated with B. Friedländer may occur in humans has recently been pointed out by Belk,[41] who found 3 in 18 cases of such infections. The clinical and pathologic findings were confused with tuberculosis.

From the cases of pseudotuberculosis we have recovered an organism which is probably in the Eberthella group (Bergey's classification). The resemblance of the lesions grossly to true tuberculosis renders it necessary to demonstrate acidfast organisms in doubtful cases, as in direct smears the organisms of pseudotuberculosis may be present in so few numbers as to escape detection.

SUMMARY.

Fifty-six cases of B. Friedländer and 36 cases of pneumococcus group 4 infections in guinea-pigs are described, the lesions included septicemia, sinusitis, otitis, pneumonia, pleurisy, pericarditis, perito-

[41] J. Infect. Dis., 1926, 38, p. 115.

nitis, metritis, cellulitis and focal abscesses. The Friedländer organism belongs to type B in Julianelle's classification, i.e., that group which cross agglutinates with type 2 pneumococcus antiserum. The pneumococcus does not agglutinate with antiserums of any of the fixed types but is related serologically by its protein fraction with human strains.

The frequency of spontaneous chronic infections in guinea-pigs is stressed and the possible effect these may have on altering the resistance of the infected animal to subsequent experimental inoculations with tubercle bacilli, is discussed. The point of entry in the majority of instances appears to be by the upper respiratory tract and middle ear and the necessity of examining the sinuses and ears at necropsy is emphasized.

Fourteen cases of pseudotuberculosis are described. Twelve of these were associated with an organism of the Eberthella group (Bergey's classification) and some experimental evidence is afforded that it is the specific infective agent and that infection can occur by feeding.

Caution is advised in diagnosing tuberculosis by gross inspection alone.

NOTE ON A PORTABLE FORM OF THE MANOMETRIC GAS APPARATUS, AND ON CERTAIN POINTS IN THE TECHNIQUE OF ITS USE.

BY DONALD D. VAN SLYKE.

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The form of the manometric apparatus shown in Figs. 1 and 2 is more convenient in several respects than the original model described by Van Slyke and Neill (1). The chief convenience is that in the present model all parts, including motor and shaking device, are attached to one simple, compact, rigid, wooden frame, so that the apparatus can be packed, shipped, and moved about from desk to desk as a unit. It is attached firmly to any table top by a carpenter's screw clamp, shown in Fig. 1.[1]

The strong, rigidly attached iron rod to which the pulley is clamped reduces vibration to a minimum and assists in making a noiseless smooth-running mechanism.

The prolongation of the glass tubing for the mercury below the level of the table top is rendered unnecessary by the use of the closed manometer.

As in the original, it will be noted that the bore of the glass manometric tubing is constricted to 1 mm. at one point above and one point below the scale. The upper constriction is to prevent mercury from striking the upper cock with destructive force; the lower one is to diminish the tendency of mercury to oscillate in the manometer while the gas volume is being reduced to the 2 or 0.5 cc. mark in the chamber.

The bottle on top of the frame holds distilled water. The lower bottle is to receive waste solutions drained out of the chamber after analyses. The most rapid and convenient way to transfer them to the

[1] The apparatus is manufactured by Eimer and Amend of New York, the Arthur H. Thomas Company of Philadelphia, the Empire Laboratory Company of New York, and by Robert Goetze, of Leipsic.

waste bottle is to force them up into the cup above the chamber and draw them over into the bottle by suction, arranged as indicated in Fig. 1. If suction is not available, however, a narrow rubber drain tube can be run directly from the curved outlet capillary of the chamber to the waste bottle.
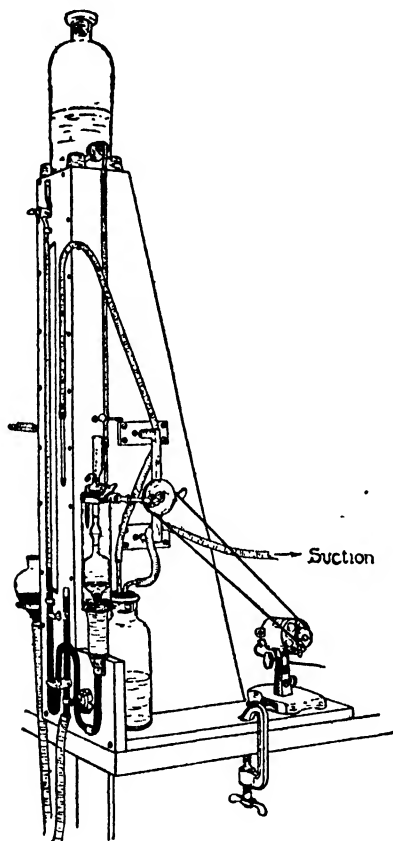
FIG. 1.

The dimensions given in the scale drawing of Fig. 2 should be adhered to. In particular the + joint in the center tubing must be located at the level indicated.

The rubber tubing for connecting the mercury-leveling bulb, and for making the inner joint connecting the gas chamber with the glass
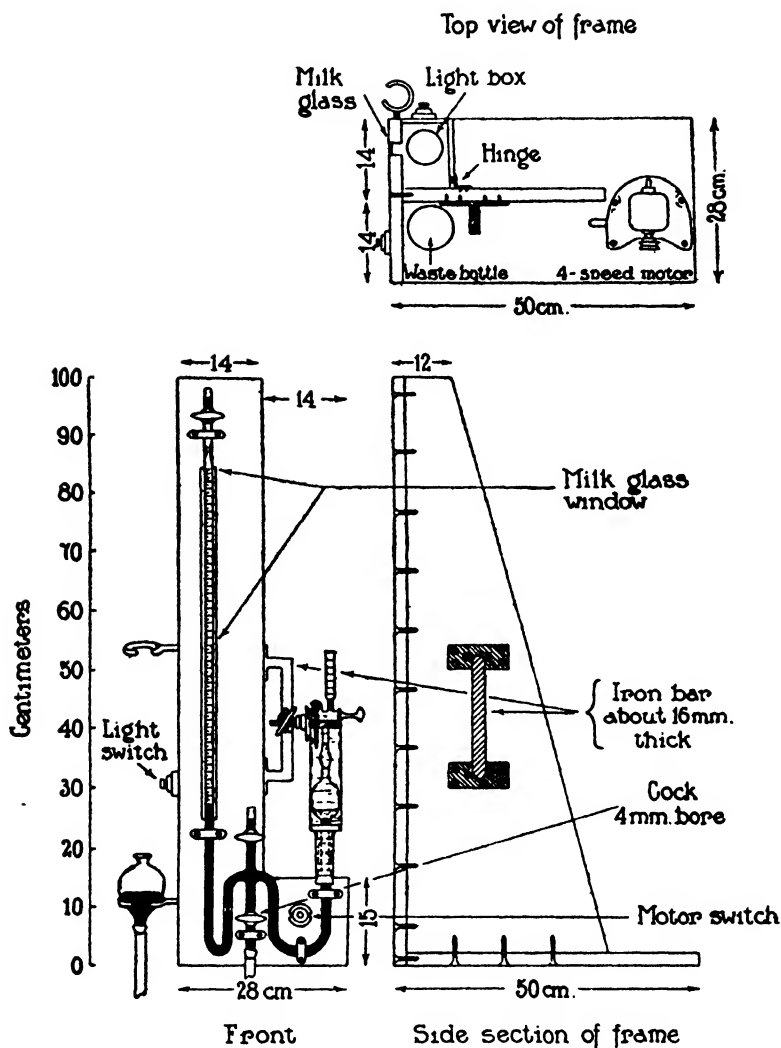
FIG. 2.

tube from the manometer (for details of joint see Fig. 2 of Van Slyke and Neill) must be the *heavy walled red nitrometer tubing*. Ordinary rubber leaks air, and also dirties the mercury with sulfide, which in turn pollutes the manometer.

The metal parts must all be of iron or steel. Brass is destroyed by the contact with mercury which is certain to occur.

As a *dehydrating agent* to wet the upper part of the manometer tube we have replaced the concentrated sulfuric acid, used by Van Slyke and Neill, by glycerol, by Kahlbaum's diethylene or trimethylene glycol, or by the methylene glycol sold as "antifreeze" for automobile radiators. These organic fluids have the advantage that they do not char the lubricant used on the cock above the manometer. The methylene and ethylene glycols are less viscous, and preferable to glycerol for that reason. Once every few days about 1 cc. is admitted into the part of the manometer tube immediately below the cock, and is then ejected. The portion that remains adherent on the walls is sufficient to absorb the vapor of moisture drawn into the manometer tube by the mercury.

Absorption of Oxygen.—For absorption of oxygen in the gas chamber the catalyst, sodium anthrahydroquinone-β-sulfonate, introduced by Fieser (2), added to the hydrosulfite, markedly accelerates the reaction.[2] We grind up in a mortar 100 gm. of sodium hydrosulfite and 10 gm. of β-sulfonate, and keep the mixture in a stoppered bottle. For preparation of the absorbent solution 10 gm. of this mixture are placed in a beaker, and 50 cc. of 1 N potassium hydroxide are poured over it. The mixture is stirred with a rod for a few seconds and quickly filtered through cotton. The filtrate is at once deaerated in the gas apparatus as described on pp. 534 and 535 of Van Slyke and Neill's paper. As noted by E. K. Marshall (personal communication) a drop of 10 per cent ferric chloride added to the solution still further accelerates its activity.

Absorption of oxygen by this solution is almost as rapid as absorption of CO_2 by alkali if the following procedure is followed: After the p_1 reading of $O_2 + N_2$ has been taken the solution in the chamber of the apparatus is lowered until a gas space of 4 or 5 cc. is obtained. The cock leading to the mercury bulb is then closed. 1.5 cc. of hydrosulfite solution are placed in the cup of the apparatus, and admitted a drop at a time. As each drop trickles down the inner wall of the chamber it absorbs oxygen, and the mercury in the

[2] The sulfonate is obtainable from the Eastman Kodak Co.

manometer falls. After a few drops have been admitted no further perceptible fall occurs. The mercury cock is then opened, and the solution in the chamber is permitted to rise as near to the stop-cock as it will, with the leveling bulb in the position shown in Figs. 1 and 2. The remainder of 1 cc. of hydrosulfite is then added, completing the absorption of the last traces of oxygen. The entire absorption can
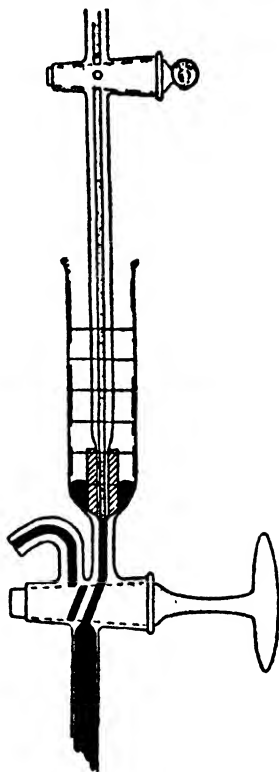
FIG. 3.

be completed in about 1 minute with the gas from 2 cc. of blood, and in considerably less with the gas from 1 cc.

For micro determinations, with 0.2 cc. portions of blood, the preliminary absorption under reduced pressure is dispensed with. A few drops of absorbent, added with the mercury cock open and the leveling bulb in the position shown in Figs. 1 and 2, complete the absorption in a few seconds.

Quantitative Transfer of Solution to the Chamber without Washing.
—With a mercury seal in the cup, solutions can be pipetted directly
into the chamber of the apparatus. A pipette is used calibrated to
deliver between two marks, and preferably provided with a stop-cock,
as shown in Fig. 3. From 0.5 to 1.0 cc. of mercury is forced up into
the cup, and the rubber-tipped point of the pipette (see p. 532 of
Van Slyke and Neill's paper) is immersed in the mercury and fitted
accurately into the bottom of the cup. The flow of solution from
the pipette into the chamber may be regulated by either the cock of
the pipette or that of the chamber.

After the pipette is withdrawn the mercury is admitted into the
chamber, forcing through before it the solution in the capillary. A
slight loss occurs, due to the fact that a droplet of solution from the
top of the capillary rises to the top of the mercury when the pipette
is withdrawn. We have determined this loss by delivering 5 cc. of
1 N sodium hydroxide into the chamber by the above technique,
then pouring 2 or 3 cc. of water into the cup, washing the mercury
about with it, and titrating the alkali with 0.02 N acid, using alizarin
sulfonate as indicator. The amount of 0.02 N acid used was 0.25
to 0.35 cc., equivalent to 0.005 to 0.007 cc. of the 1 N alkali. With
properly fitting pipette tip and rubber ring, therefore, the loss in the
above method of delivery can be kept within these limits. The tech-
nique can accordingly be used for the quantitative transfer of portions
of 2 cc. or greater volume, without significant loss. For quantitative
transfer of smaller portions washing must be used.

The portable apparatus above described was developed with the
assistance of John Plazin. A marked mechanical improvement in
the form of a well machined ball bearing pulley, reducing vibration
to a minimum, was introduced by Mr. Walter Eimer of the firm of
Eimer and Amend.

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CARBON DIOXIDE FACTORS FOR THE MANOMETRIC BLOOD GAS APPARATUS.

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When CO_2 is determined in either the original volumetric apparatus of Van Slyke or the present manometric apparatus the gas is extracted from solution by shaking the latter in the evacuated chamber until equilibrium between gas phase and liquid phase is reached. Mercury is then admitted into the chamber until the gas phase is reduced from 43 or more cc. to 2 cc. or less before the gas is measured. During this reduction of volume the tension of CO_2 in the gas phase is proportionately increased, and in consequence a small part of the CO_2 passes back into the solution. Van Slyke and Stadie (1921) found that this reabsorption under the conditions of analysis in the volumetric apparatus amounted quite constantly to 1.7 per cent of the total CO_2 present in the system, and to correct for it introduced the i factor 1.017 into their calculation factors. Van Slyke and Neill (1924) for the manometric apparatus found an i factor of 1.014 when the gas phase in the 50 cc. apparatus was reduced to 2 cc., 1.03 when it was reduced to 0.5 cc.

An increasing refinement of technique in the preparation, handling, and analysis of standard carbonate solutions has made it possible to determine the i factor for the manometric apparatus with increased precision. Both direct determinations of reabsorbed CO_2, and estimations of it from the proportion of expected CO_2 obtained, indicate that for the above conditions the i factor has the values 1.017 and 1.037 instead of the former values 1.014 and 1.030.

Furthermore our results indicate that the value 22.26 liters, determined by Guye and Pintza (1908) for the molecular volume of CO_2 measured at 0°, 760 mm. pressure, is exact, rather than the conventional 22.4 liters. Van Slyke and Neill employed the value 22.4 in

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their calculations, and intentionally incorporated any inaccuracy due to the use of this figure in their i factors. The total effect of changing the i factor from 1.014 to 1.017 and the molecular volume from 22.40 to 22.26 liters is to increase by approximately 1 per cent the factors of Van Slyke and Neill for calculating millimolar CO_2 concentrations. Volume per cent factors are affected only by the change in i, and are increased by but 0.3 per cent.

The results of analyses of standard sodium carbonate solutions, on which the work in this paper is based, have been further verified by determinations of the CO_2 evolved by permanganate oxidation of oxalic acid in connection with gasometric calcium analyses (Van Slyke and Sendroy, 1926). The authors have checked each others results. In this paper the results with carbonate solutions only will be presented.

Calculations.

The fundamental equation, on which all calculations in the manometric gas apparatus are based, is the following (Equation 4 of Van Slyke and Neill):

$$(1) \qquad V_{0°, 760} = a \; \times \; \frac{P}{760} \times \frac{1}{1 + 0.00384\, t} \times \left(1 + \frac{S}{A - S}\, \alpha'\right) \times i$$

| Observed gas volume. | Correction for pressure. | Correction for temperature effect on gas and on Hg in manometer. | Correction for unextracted gas, according to Henry's law. | Correction for reabsorbed gas. |

We may combine all the factors except P into a single factor, by which P is multiplied to obtain $V_{0°,\ 760}$. Such a combined factor, with given a, A, and S, is dependent only on the temperature.

$$(2) \qquad V_{0°, 760} = P \times \left[\frac{i\, a}{760\, (1 + 0.00384\, t)} \times \left(1 + \frac{S}{A - S}\, \alpha'\right) \right]$$

$$= P \times \text{[volume factor]}$$

$V_{0°,\ 760}$ = volume of gas, measured at 0°, 760 mm., in the solution analyzed; P = partial pressure of the extracted gas read on the manometer; i = the correction factor for reabsorption; a = the

volume to which the gas phase is reduced after extraction and at which the final pressure readings are made; t = temperature in °C.; S = total volume of solution from which the gas is extracted; A = the volume of chamber occupied by gas and solution during extraction; $A - S$ = volume of gas phase during extraction; α' is the Henry distribution coefficient of the gas between gas and liquid phases. $\alpha' = \alpha \times \dfrac{T}{273}$, where α is the Bunsen solubility coefficient, T the absolute temperature. For illustration of the values a, A, S, $A - S$, see Fig. 1 of Van Slyke and Neill.

Dividing the volume factor in Equation 1 by 22.26, the volume in cc. of 1 milligram molecule (= 1 millimol = 44 mg.) of CO_2 reduced to 0°,760 mm., we have

(3) $\qquad$ m$_M$ CO_2 in the portion of solution analyzed

$$= P \times \left[\frac{i\,a}{16,918\,(1 + 0.00384\,t)} \times \left(1 + \frac{S}{A - S}\,\alpha'\right) \right]$$

Multiplying the volume factor in Equation 2 by 1.9766, the weight in mg. of 1 cc. of CO_2 at 0°,760 mm., we have

(4) $\qquad$ Mg. $CO_2 = P \times \left[\dfrac{1.9766\,i\,a}{760\,(1 + 0.00384\,t)} \times \left(1 + \dfrac{S}{A - S}\,\alpha'\right) \right]$

To change to terms indicating CO_2 as volumes per cent or millimols per liter in the solution analyzed the factors in Equations 2 and 3 are multiplied by $\dfrac{100}{\text{cc. sample}}$ and $\dfrac{1000}{\text{cc. sample}}$, respectively, and yield the following:

(5) Vol. per cent $CO_2 = P \times \left[\dfrac{0.13159\,i\,a}{(\text{cc. sample})\,(1 + 0.00384\,t)} \times \left(1 + \dfrac{S}{A - S}\,\alpha'\right) \right]$

$\qquad\qquad = P \times$ vol. per cent factor

and

(6) m$_M$ CO_2 per liter $= P \times \left[\dfrac{0.05911\,i\,a}{(\text{cc. sample})\,(1 + 0.00384\,t)} \times \left(1 + \dfrac{S}{A - S}\,\alpha'\right) \right]$

$\qquad\qquad = P \times$ m$_M$ per liter factor

The observed reading, P, is multiplied in each case by a total factor (in brackets) to calculate in the terms desired the amount of CO_2 present in the sample of solution analyzed (by Equations 1 to 4), or the concentration of CO_2, in volumes per cent or millimols per liter, in the solution from which the sample for analysis was taken (Equations 5 and 6).

The factor i was determined by direct estimation, in a way described later, and was also calculated from analyses of standard solutions of Na_2CO_3 according to Equation 7, which is Equation 8 of Van Slyke and Neill with the factor 0.05911 in place of 0.0587.

$$(7) \qquad i = \frac{1}{P} \times \frac{[CO_2] \text{ (cc. sample)}}{0.05911 \, a} \times \frac{1 + 0.00384 \, t}{1 + \dfrac{S}{A - S}},$$

$[CO_2]$ represents millimols of CO_2 present per liter of the standard solution.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Preparation of Standard Na₂CO₃ Solutions.*</div>

The purest $NaHCO_3$ obtainable was heated in a furnace for 3 hours at a temperature of 290–300°C. After slight cooling, the resultant Na_2CO_3 was placed in a desiccator in a bottle, which was opened only when a solution was to be prepared.

The water used to prepare the standard solutions was redistilled from alkaline or acid potassium permanganate and received in flasks protected from atmospheric CO_2 by soda-lime tubes. The distillate, however, always contained several hundredths of a volume per cent of CO_2. The preparation and preservation of really CO_2-free water is, in fact, impossible without extraordinary precautions. Instead of attempting it we have in each blank analysis of the reagents substituted for the sodium carbonate solution an equal volume of the water with which it was prepared, so that the result of the blank included the corrections both for the reagents and for the CO_2 in the water. The P_{CO_2} readings observed in the blanks were subtracted from those obtained in analyses of the standard carbonate solutions.

The carbonate solutions, as soon as prepared, were transferred to Pyrex glass tubes described by Austin *et al.* (1922), and kept over
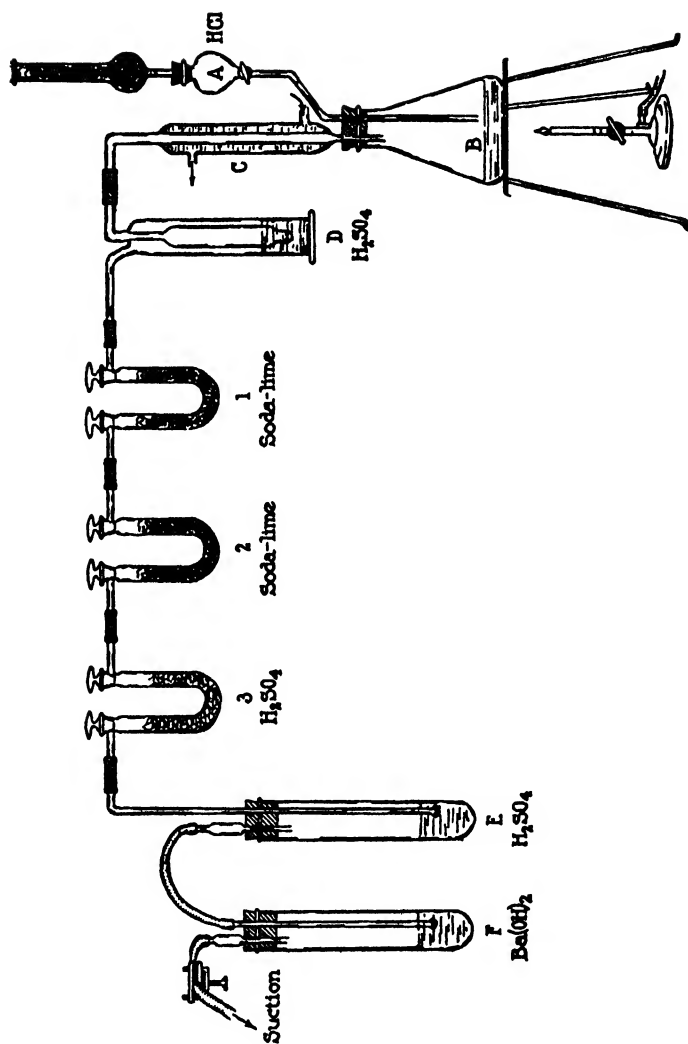
FIG. 1.

mercury and out of contact with the atmosphere. In this condition they could be kept for days without change in CO_2 content. The portions for analysis were drawn into stop-cock pipettes as described under "Sampling Blood" on p. 131 of the Austin *et al.* paper.

In order to control the purity of the carbonate and the accuracy of the standard solutions, they were analyzed as described below, by two gravimetric and one titrimetric method.

1. Carbon dioxide in both solid Na_2CO_3 and standard Na_2CO_3 solutions was determined gravimetrically by the well known method of driving the gas out of solution by acidifying, heating, and passing a stream of CO_2-free air. The evolved CO_2 was absorbed and weighed in soda-lime tubes.

A known amount of standard solution, or of solid carbonate and analyzed water, was placed in the flask B (Fig. 1), which was then connected with the rest of the closed system as indicated. A protecting soda-lime tube was attached to the funnel, A, which dropped dilute HCl into the flask. The evolved CO_2 passed through the condenser, C, the wash bottle, D, containing concentrated sulfuric acid, two weighed U-tubes containing soda-lime (1 and 2), and a third containing pumice stone saturated with sulfuric acid (3), then through the aeration tubes, E and F, containing sulfuric acid and clear barium hydroxide solution, respectively. A slow current of air was drawn through the train by suction, the evolution of gas being regulated by means of the stop-cocks on the funnel and the pinch-clamp on the suction tube. After addition of the acid heat was applied to the flask, and the temperature of the solution was very slowly increased to boiling. The stem of the funnel was then lowered beneath the surface of the boiling liquid, and air was drawn through the system until all the CO_2 was transferred to the soda-lime tubes. The barium hydroxide solution at the end of the train indicated any escape of CO_2. When this occurred the analysis was discarded. The three U-tubes were carefully cleaned with ether, wiped dry with lens paper, and allowed to come to room temperature before each weighing. Blank analyses done on the same day, on the same water used for the solution, served to correct for the amount of CO_2 in the water and in the air of the flask and wash bottle.

Some typical results are given in Table I.

2. To determine the sodium contents, portions of the same solutions of Na_2CO_3 were measured out into platinum dishes and dilute H_2SO_4 was added, with care to avoid spattering. The solutions were concentrated as far as possible on the water bath and the free H_2SO_4 was driven off by heating, first on a hot plate, and finally with a

TABLE I.

Gravimetric Determination of CO_2 in Standard Sodium Carbonate Solution.
Solution contained 10.5753 gm. Na_2CO_3 per liter.

Volume of sample.	Calculated weight of CO_2 in sample.	Weight of CO_2 found minus average blank of 0.0006 gm. (3 analyses).	Per cent of theoretical.
cc.	*gm.*	*gm.*	
100	0.4390	0.4392	100.05
100	0.4390	0.4385	99.89
100	0.4390	0.4389	99.98
Average..			99.97

TABLE II.

Gravimetric Determination of Sodium in Standard Sodium Carbonate Solution as Barium Sulfate.

Na_2CO_3 in solution.	*a* BaSO_4 found.	0.1 N NaOH to neutralize free H_2SO_4.	*b* BaSO_4 equivalent to free H_2SO_4.	*a − b* BaSO_4 from Na_2SO_4.	Na found in Na_2CO_3.	Per cent of theoretical Na found.
gm.	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	
0.2115	0.4728	0.57	0.0067	0.4661	43.42	100.07
0.2115	0.4741	0.67	0.0078	0.4663	43.44	100.11
0.2027	0.4944	4.09	0.0477	0.4467	43.42	100.07
0.2027	0.4949	3.91	0.0456	0.4473	43.48	100.20
Average...					43.44	100.11
Theoretical..					43.39	

micro burner. The sodium sulfate was dissolved in water, and any excess H_2SO_4 not driven off was detected and measured by titration with standard NaOH solution. The solutions were then acidified with HCl, and the sulfate was precipitated hot with $BaCl_2$. Table II indicates the results obtained.

3. As a further check, on the accuracy of the standard solutions, the latter were also titrated. An excess of 0.1 N HCl made from constant boiling acid prepared according to Hulett and Bonner (1909) was added to the carbonate solution, which was then boiled to drive off CO_2. After cooling, the excess HCl was titrated back with 0.1 N

TABLE III.

Determination of Reabsorption Correction, i, by Analysis of a Standard Na_2CO_3 Solution.

A = 100.0 cc. S = 7.00 cc. a = 4.009 cc.   2 cc. samples of Na_2CO_3 solution.   Blank = 2.0 mm. = c.

Solution.	Na_2CO_3 concentration. $[CO_2]$	p_1	p_2	$P_{CO_2} = p_1 - p_2 - c$	Temperature.	c Calculated by Equation 7.	Deviation from average of Tables III and IV.
	mM	$mm.$	$mm.$	$mm.$	$°C.$		
A	49.89	652.3	231.0	419.3	21.9	1.020	+0.003
		651.5	231.0	418.5	21.0	1.017	0.000
		650.6	230.8	417.8	20.7	1.017	0.000
		651.4	232.0	417.4	21.0	1.020	+0.003
		655.1	234.0	419.1	21.8	1.020	+0.003
		658.0	235.0	421.0	22.4	1.018	+0.001
		657.4	234.8	420.6	22.0	1.017	0.000
		656.6	234.6	420.0	22.0	1.019	+0.002
		658.5	235.1	421.4	22.2	1.016	−0.001
		660.0	237.0	421.0	22.8	1.020	+0.003
		660.7	236.6	422.1	23.0	1.019	+0.002
		663.8	237.6	424.2	23.8	1.018	+0.001
		666.7	238.8	425.9	24.3	1.016	−0.001

3 cc. samples of Na_2CO_3 solution.   Blank = 1.8 mm. = c.

Solution.	Na_2CO_3 concentration. $[CO_2]$	p_1	p_2	$P_{CO_2} = p_1 - p_2 - c$	Temperature.	c Calculated by Equation 7.	Deviation from average of Tables III and IV.
E	33.26	688.6	259.6	426.5	24.8	1.017	0.000
		688.5	258.5	427.5	24.8	1.015	−0.002
		688.5	259.0	427.0	24.8	1.016	−0.001

NaOH which had been standardized against the same HCl solution. Various analyses at different times and on different solutions indicated a content of Na_2CO_3 varying between 99.7 per cent and 100.2 per cent of the theoretical.

From the foregoing, it is apparent that the Na_2CO_3 used was as nearly pure as could be ascertained by methods approaching one

per thousand in accuracy, and hence was a proper basis for standard solutions.

Determination of the i Factor.

To determine the i factor, standard sodium carbonate solutions of varying concentration were analyzed by the Van Slyke-Neill

TABLE IV.

Determination of Reabsorption Correction, i, by Analysis of a Standard Na_2CO_3 Solution.

A = 50 cc. S = 3.5 cc. a = 2.002 cc.   1 cc. samples of Na_2CO_3 solution. Blank = 0.7 mm.

Solution.	Na_2CO_3 concentration. [CO$_2$]	p_1	p_2	P_{CO_2} = $p_1 - p_2 - 0.7$	Temperature.	i Calculated by Equation 7.	Deviation from average of Tables III and IV.
	mM	*mm.*	*mm.*	*mm.*	°C.		
B	49.99	492.0	71.5	419.8	21.2	1.018	+0.001
		491.6	72.0	418.9	21.2	1.020	+0.003
		491.4	71.3	419.4	21.2	1.019	+0.002
		491.6	71.2	419.7	21.3	1.019	+0.002
		492.5	71.8	420.0	21.4	1.019	+0.002
		491.5	71.4	419.4	21.2	1.019	+0.002
C	15.00	201.4	73.5	127.2	22.5	1.015	−0.002
		201.1	73.3	127.1	22.7	1.017	0.000
		202.2	74.0	127.5	23.0	1.016	−0.001
.		202.3	74.0	127.6	23.2	1.016	−0.001
		202.4	74.0	127.7	23.2	1.015	−0.002
D	30.00	336.9	83.4	252.8	21.5	1.016	−0.001
		337.7	84.0	253.0	21.5	1.015	−0.002
		336.3	83.5	252.1	21.1	1.017	0.000
		336.4	83.5	252.2	20.8	1.015	−0.002

technique during the course of 4 months in several different blood gas apparatus, of different capacity, with open and closed manometers. From the results the i correction was estimated by Equation 7. Within the limits of error, the same results were given by all the different apparatus.

The technique followed for reducing the gas volume from $(A - S)$ to a cc. was that described on p. 533 of Van Slyke and Neill's paper.

The mercury cock was opened with a smooth, gradual motion, the mercury was permitted to rise fairly rapidly through the wide, cylindrical part of the chamber, and was gradually retarded as the meniscus of the solution approached the a mark. When the latter was reached the movement had been so regarded that *no oscillation* of the solution in the chamber occurred after the mercury cock was closed. As pointed out on p. 26 of Van Slyke and Stadie's paper, such oscillation can cause a gross increase in reabsorption of CO_2. The time taken for the volume reduction was 30 to 40 seconds in the

TABLE V.

Determination of Reabsorption Correction, i, by Analysis of a Standard Na_2CO_3 Solution under Conditions for Micro Analysis in 100 Cc. Apparatus with
a = 1 Cc.

$A = 100$ cc. $S = 7.00$ cc. $a = 1.000$ cc. 3 cc. samples of Na_2CO_3 solution. Blank = 6.4 mm.

Solution.	Na_2CO_3 concentration [CO_2]	p_1	p_2	$P_{CO_2} = p_1 - p_2 - 6.4$ blank.	Temperature.	i Calculated by Equation 7.	Deviation from average of Tables V and VI.
	mM	$mm.$	$mm.$	$mm.$	$°C.$		
M	8.04	697.2	287.5	403.3	23.2	1.034	−0.003
		696.5	287.9	402.2	23.4	1.038	+0.001
		699.6	208.7	404.5	23.7	1.034	−0.003
		696.3	285.5	404.4	23.8	1.035	−0.002
		695.1	286.4	402.3	23.7	1.040	+0.003
		694.8	285.3	403.1	23.5	1.036	−0.001
		694.3	284.5	403.4	23.6	1.036	−0.001
		644.1	283.4	403.8	23.7	1.036	−0.001

50 cc. apparatus, 45 to 55 seconds in the 100 cc. apparatus. If much more time is taken, reabsorption is increased. However, it is our experience that an analyst, after acquiring facility in the use of the apparatus, automatically learns to admit the mercury at rates within the necessary limits. The p_1 manometer reading is made as soon as convenient after the gas has been brought to a cc. volume. The meniscus will stand thus for some seconds without measurable effect on p_1, but longer standing results in significant increase in reabsorption.

Some typical analyses are given in Tables III to VI. The average of the results in Tables III and IV gives a value of 1.017 for i when readings are made with a gas volume 2.0 cc. in the 50 cc. apparatus or of 4.0 cc. in the 100 cc. apparatus. Tables V and VI indicate an average value of 1.037 for i when readings are made in the 50 cc. apparatus with a gas volume of 0.5 cc., or in the 100 cc. apparatus with a gas volume of 1.0 cc.

TABLE VI.

Determination of Reabsorption Correction, i, by Analysis of a Standard Na_2CO_3 Solution under Conditions for Micro Analysis in 50 Cc. Apparatus with $a = 0.5$ Cc.

$A = 50$ cc. $S = 2.0$ cc. $a = 0.500$ cc. 1 cc. samples of Na_2CO_3 solution. Blank $= 3.7$ mm.

Solution.	Na_2CO_3 concentration. $[CO_2]$	p_1	p_2	$P_{CO_2} = p_1 - p_2 - 3.7$	Temperature.	i Calculated by Equation 7.	Deviation from average of Tables V and VI.
	mM	$mm.$	$mm.$	$mm.$	$°C.$		
F	7.50	382.0	122.7	255.6	22.5	1.040	+0.003
		379.8	120.0	256.1	22.5	1.038	+0.001
		382.1	121.2	257.2	22.7	1.035	−0.002
		381.3	121.8	255.8	23.0	1.042	+0.005
		382.9	121.4	257.8	22.9	1.033	−0.004
		381.2	120.5	257.0	23.0	1.037	0.000
G		368.9	108.9	256.3	21.7	1.034	−0.003
		366.9	107.3	255.9	21.8	1.036	−0.001
		366.2	107.3	255.2	21.7	1.038	+0.001
		368.3	108.0	256.6	22.6	1.037	0.000
		365.5	105.5	256.3	23.0	1.040	+0.003
		369.6	107.8	258.1	23.1	1.033	−0.004

In order further to verify these i factors the following method was employed to obtain a more direct determination of the reabsorbed CO_2. With the Harington-Van Slyke (1924) apparatus, a standard solution of Na_2CO_3 was analyzed in such a way as to determine (a) the amount of CO_2 left in solution after the first extraction and attainment of equilibrium; (b) this amount of CO_2 *plus* the amount reabsorbed during release of the vacuum and reduction of the gas to the volume at which the pressure was read. The difference between (a) and (b) gives the amount of CO_2 reabsorbed.

The quantity (a) was determined as follows: 0.15 cc. of 10 per cent lactic acid and 2.35 cc. of water were placed in the chamber of a 50 cc. Harington-Van Slyke apparatus and feed of all gases by twice extracting the solution and ejecting the gases. About 1 cc. of the extracted solution was forced up into the cup, and a 1 cc. sample of standard carbonate solution was run from a stop-cock pipette into the chamber of the apparatus, with the technique detailed on p. 532 of Van Slyke and Neill's paper. The acid solution in the cup was returned to the chamber, the latter was evacuated, and the CO_2 was extracted. With the lower stop-cock of the Harington-Van Slyke chamber closed, the upper stop-cock was opened, and air was allowed to enter to atmospheric pressure. The CO_2 gas in the chamber was thus diluted with air without changing the CO_2 tension from that obtained at equilibrium at the end of the extraction. Mercury was then admitted into the chamber through the lower cock till all the air-CO_2 gas mixture was expelled through the upper cock, the solution being retained in the chamber. In this manner the CO_2 tension of the gas phase above the solution was kept constant, so that there was no reabsorption of CO_2 during ejection of the gases. The solution was again extracted, and the pressure was read at the 2.0 or the 0.5 cc. mark, giving p_1. The CO_2 was then absorbed with 2 drops of 20 per cent NaOH, and the reading p_2 was made. Using the factors for a 1 cc. sample in Table X of this paper, the unextracted CO_2 was calculated, in terms of millimols per liter of the original sample analyzed. Table VII shows the good agreement obtained between the CO_2 thus determined and that calculated to be left in the solution after the first extraction, thereby confirming the validity of Henry's law under the conditions of the analysis. The calculated unextracted CO_2 values in the last column of the table were obtained by multiplying the original CO_2 content of the solution analyzed, *viz.* 30.08 mM, by the factor $\dfrac{S}{A-S} \, \alpha'$.

For the direct determination of (b), *unextracted plus reabsorbed CO_2,* the technique of the usual CO_2 determined was carried through up to the point where the meniscus of the extracted solution was raised to the 2 cc. mark in the Harington-Van Slyke chamber. The lower stop-cock of the chamber was then quickly closed, and a suction tube

attached to a pump was pressed into the bottom of the cup. The upper stop-cock was then opened, whereupon the suction immediately drew the gas out of the space above the solution in the chamber. By repeatedly pressing the suction tube into the bottom of the cup for a few seconds and then removing it, the replacement of the CO_2 in the gas space of the chamber by air was made complete. Since the lower cock of the chamber was closed, the meniscus of the solution

TABLE VII.

Direct Determination of Unextracted CO_2 in a Standard Sodium Carbonate Solution

Solution.	p_1	p_2	$P_{CO_2} =$ $p_1 - p_2$	Temperature.	Unextracted CO_2.[*]	
					Observed.	Calculated as $30.08 \dfrac{S}{A-S}\alpha'$

$A = 50$ cc. $S = 3.5$ cc. $a = 2.003$ cc. 1 cc. samples of Na_2CO_3 solution. Concentration $= 30.08$ mM per liter, including CO_2 content of water.

	mm.	*mm.*	*mm.*	*°C.*	*mM*	*mM*
H	182.9	166.3	16.6	23.3	1.95	1.96
	182.3	166.1	16.1	25.0	1.88	1.88
	187.0	171.0	16.0	24.9	1.87	1.88
	181.2	165.8	15.4	24.9	1.80	1.88
	181.6	165.3	16.3	24.6	1.91	1.90
	179.6	162.1	17.5	20.8	2.09	2.09

$= 50$ cc. $S = 3.5$ cc. $a = 0.500$ cc. 1 cc. samples of Na_2CO_3 solution. Concentration $= 40.00$ mM per liter including CO_2 content of water.

J	300.3	214.2	86.1	23.8	2.57	2.58
	300.3	216.0	84.3	24.0	2.52	2.56
	288.0	203.0	85.0	23.8	2.54	2.58

[*] In mM per liter of original solution.

in the narrow tube of the chamber at the 2.0 cc. mark remained perfectly quiet, and there was no measurable loss of previously unextracted CO_2 from the solution. Finally the air was expelled from the chamber by admission of mercury from below, and the CO_2 remaining in the solution was determined in the usual manner, by extracting, reducing the volume to 2.0 cc., and measuring the pressure before and after absorption of the CO_2 gas with 2 drops of 20 per

cent NaOH. The reabsorbed CO_2 was computed as the difference between the calculated volume value of (a) and the determined value of (b), the accuracy of the factor $\dfrac{S}{A-S}$ for calculating (a) having been demonstrated by the data in Table VII. The results are given in Table VIII. They indicate an average reabsorption equal to

TABLE VIII.

Direct Determination of Reabsorbed CO_2 in a Standard Sodium Carbonate Solution.

Solution.	p_1	p_2	$P_{CO_2} = p_1-p_2-c$	Temperature.	a CO₂ unextracted + reabsorbed.	b CO₂ unextracted calculated as $30.08 \times \dfrac{S}{A-S}a'$	$a-b$ CO₂ reabsorbed.	Proportion of total CO₂ reabsorbed.	Deviation from average.
A = 50 cc. S = 3.5 cc. a = 2.003 cc. 1 cc. samples of Na₂CO₃ solution. Concentration = 30.08 mM per liter including CO₂ content of water.
	mm.	mm.	mm.	°C.	mM	mM	mM		
K	185.3	163.9	21.4	24.3	2.51	1.91	0.60	0.020	+0.002
	184.2	163.2	21.0	24.9	2.45	1.88	0.57	0.019	+0.001
	185.2	163.7	21.5	24.3	2.51	1.91	0.60	0.020	+0.002
	184.0	163.7	20.3	24.8	2.37	1.88	0.49	0.016	−0.002
	180.4	157.3	23.1	18.9	2.78	2.19	0.59	0.019	+0.001
A = 50 cc. S = 3.5 cc. a = 0.500 cc. 1 cc. samples of Na₂CO₃ solution. Concentration = 40.00 mM per liter including CO₂ content of water.
L	316.0	204.5	111.5	24.0	3.33	2.56	0.77	0.019	+0.001
	314.2	205.0	109.2	23.7	3.27	2.58	0.69	0.017	−0.001
	301.5	192.2	109.3	24.0	3.27	2.56	0.71	0.018	0.000
	298.0	190.5	107.5	23.5	3.22	2.59	0.63	0.016	−0.002
Average..								0.018	±0.0013

0.018 of the CO_2 in the analyzed sample of solution, hence an *i* factor of 1.018. The latter confirms within the limit of error the value 0.107 obtained from the results in Tables III and IV.

Computation of Tables of Factors for Use in CO_2 Determinations.

With the *i* factors above determined, total factors for use in analyses covering the ordinary range of conditions were computed by Equa-

tions 4, 5, and 6. The factors are given in Tables IX to XI. Their exactness has already been demonstrated by the analyses on which they are based in Tables III to VI.

TABLE IX.

Factors by Which Millimeters P_{CO_2} are Multiplied to Give Milligrams CO_2 in the Sample Analyzed. 50 Cc. Apparatus.

Tempera-ture.	S = 2.0 cc.		S = 3.5 cc.		S = 7.0 cc.	
	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017	a = 0.5 cc. i = 1.037	a = 0.2 cc. i = 1.017	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017
°C.						
10	0.001366	0.005357	0.001419	0.005570	0.001560	0.006121
11	58	29	11	34	48	6068
12	52	03	01	01	34	17
13	46	5278	1394	5469	22	5969
14	38	50	86	36	10	22
15	32	26	78	06	1498	5878
16	26	00	70	5374	86	31
17	20	5175	62	45	75	5787
18	12	51	56	17	65	44
19	07	27	48	5287	55	04
20	01	06	40	62	45	5665
21	1297	5084	34	34	35	29
22	91	60	26	06	25	5590
23	85	36	20	5179	15	50
24	79	15	14	53	05	15
25	73	4993	07	27	1397	5479
26	67	71	01	02	88	42
27	63	51	1295	5078	80	08
28	57	30	89	54	70	5376
29	51	10	83	30	62	44
30	47	4890	77	07	54	13
31	41	70	71	4985	48	5285
32	37	51	65	63	40	56
33	31	31	59	42	32	26
34	27	13	53	20	26	5198

The same i values have been used to calculate the factors for analyses with different S values, since it has been shown by Van Slyke and Stadie that under the conditions of the analyses reabsorbed CO_2 is

TABLE X.

Factors by Which Millimeters P_{CO_2} are Multiplied to Give mM CO_2 per Liter in Solution Analyzed. 50 Cc. Apparatus.

Temperature.	Sample = 0.2 cc. S = 2.0 cc. a = 0.5 " i = 1.037	Sample = 1.0 cc.					
		S = 2.0 cc.		S = 3.5 cc.		S = 7.0 cc.	
		a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017
°C.							
10	0.1552	0.0310	0.1218	0.0323	0.1266	0.0355	0.1391
11	44	09	11	21	58	52	79
12	36	07	05	19	50	49	67
13	29	06	0.1199	17	43	46	57
14	21	04	93	15	36	43	46
15	14	03	88	13	29	41	35
16	07	01	82	11	22	38	25
17	0.1499	00	76	10	15	35	15
18	92	0.0298	71	08	08	33	06
19	86	97	66	06	02	31	0.1297
20	79	96	60	05	0.1196	28	88
21	72	94	55	03	90	26	79
22	66	93	50	02	83	24	70
23	59	92	45	00	77	22	62
24	53	91	40	0.0299	71	19	53
25	46	89	35	97	65	17	45
26	40	88	30	96	60	15	37
27	34	87	25	94	54	13	29
28	28	86	20	93	49	11	22
29	22	84	16	91	43	10	15
30	16	83	11	90	38	08	08
31	11	82	07	89	33	06	01
32	05	81	02	88	28	05	0.1195
33	00	80	0.1098	86	23	03	88
34	0.1394	79	94	85	18	01	82

To obtain mM per liter factors for a sample other than 1 cc., divide the above factors for 1 cc. by the cc. of sample analyzed: *e.g.*, for a 2 cc. sample, S, A, and *a* being the same, the factors are one-half of those for a 1 cc. sample.

To calculate milligram molecules of CO_2 in the actual portion of solution analyzed, use the above mM per liter factor for 1 cc. sample, divided by 1000.

TABLE XI.

Factors by Which Millimeters P_{CO_2} are Multiplied to Give Volumes Per Cent CO_2 in Solution Analyzed. 50 Cc. Apparatus.

Temperature.	Sample = 0.2 cc. S = 2.0 cc. a = 0.5 " i = 1.037	Sample = 1.0 cc.					
		S = 2.0 cc.		S = 3.5 cc.		S = 7.0 cc.	
		a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017	a = 0.5 cc. i = 1.037	a = 2.0 cc. i = 1.017
°C.							
10	0.3454	0.0691	0.2710	0.0718	0.2818	0.0789	0.3097
11	37	87	0.2696	14	00	83	70
12	19	84	83	09	0.2783	76	44
13	03	81	70	05	67	70	20
14	0.3386	77	56	01	50	64	0.2996
15	70	74	44	0.0697	35	58	74
16	54	71	31	93	19	52	50
17	38	68	18	89	04	46	28
18	22	64	06	86	0.2690	41	06
19	07	61	0.2594	82	75	36	0.2886
20	0.3292	58	83	78	62	31	66
21	78	56	72	75	48	26	48
22	63	53	60	71	34	21	28
23	48	50	48	68	20	16	08
24	34	47	37	65	07	11	0.2790
25	20	44	26	61	0.2594	07	72
˙26	06	41	15	58	81	02	53
27	0.3193	39	05	55	69	0.0698	36
28	79	36	0.2494	52	57	93	20
29	66	33	84	49	45	89	04
30	53	31	74	46	33	85	0.2688
31	40	28	64	43	22	82	74
32	28	26	54	40	11	78	59
33	15	23	44	37	00	74	44
34	03	21	35	34	0.2489	71	30

To obtain factor for a sample other than 1 cc., divide the above factors for 1 cc. by the cc. of sample analyzed: *e.g.*, for a 2 cc. sample the factors are one-half of those for 1 cc.

To calculate cc. of CO_2, measured at 0°, 760 mm., in the actual portion of solution analyzed, use the above volume per cent factors for 1 cc. samples divided by 100.

a constant proportion of the amount of CO_2 present, and is independent of the volume of water solution on the surface of the mercury, when the mercury is completely covered.

When the 100 cc. apparatus is used, the factors for the 50 cc. apparatus may be applied provided the relationships, *sample: a:S:A* are the same. *E.g.*, for analysis of a 2 cc. sample in the 100 cc. apparatus with $S = 7$ cc. and $a = 4$ cc., one uses the same factors in calculating volumes per cent CO_2 as in analysis of a 1 cc. sample in the 50 cc. apparatus, with $S = 3.5$ cc. and $a = 2$ cc. For conditions other than those defined by the tables, one may readily calculate proper factors by means of Equation 4, 5, or 6. The values for α and $\dfrac{1}{1 + 0.00384\,t}$ required for calculating such a table of factors are given in Table I of Van Slyke and Neill's paper, and the manner of simplifying the calculations by combining constants is described at the bottom of pp. 541 and 542 of the same paper.

SUMMARY.

The factors for calculating the results of CO_2 analyses in the manometric gas apparatus have been submitted to rigid experimental tests with standard Na_2CO_3 solution, the accuracy of which was controlled to approximately 1 part per 1000 by three independent modes of analysis, gravimetric Na and CO_2 determinations, and alkalimetric titrations.

On the basis of results obtained with these standard solutions tables of analytical factors have been computed with increased precision.

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AN INFECTIOUS GRANULAR VAGINITIS OF COWS.

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PLATE 15.

An infectious disease of cows characterized by an acute inflammation
of the vaginal mucosa and terminating in the formation of raised red
nodules studding the mucosa has been recognized in many parts of
the world. Hess[1] reported that in certain sections of Switzerland
over 60 per cent of the cows suffered with the disease. It is one of
the common disorders of cows in this country.

Ostertag,[2] Hecker,[3] and others succeeded in cultivating a Gram-negative
streptococcus from the mucopurulent exudate. The organism was described
as extracellular and occurred in chains of from 6 to 9. In many instances it was
associated with staphylococci and *B. coli*. Ostertag inoculated the vagina of
cattle, sheep, goats, pigs, and mares with the streptococcus and reproduced the
disease. He points out that Raebiger,[4] Jüterbock,[5] and others reproduced the
disease in cows by intravaginal inoculation with a similar streptococcus. Blaha[6]
observed in a series of cases bodies embedded in the epithelial cells similar in many
respects to those observed in trachoma, which led him to believe that it was a
Chlamydozoa infection.

Relatively little concerning the etiology of vaginal infections has been pub-
lished in this country. Starr[7] noted that the nodules resulted from hyperplasia
of the lymph follicles as the result of irritation. He succeeded in cultivating a
streptococcus of the *viridans* type from the exudate.

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[1] Hess, cited by Ostertag.[2]

[2] von Ostertag, R., in Kolle, W., and von Wassermann, A., Handbuch der
pathogenen Mikroorganismen, Jena, 2nd edition, 1913, vi, 269.

[3] Hecker, *Berl. tierärztl. Woch.*, 1900, 445.

[4] Raebiger, W., *Berl. tierärztl. Woch.*, 1907, 254.

[5] Jüterbock, K., *Z. Tiermed.*, 1909, xiii, 354.

[6] Blaha, E. H., *Berl. tierärztl. Woch.*, 1909, xxv, 879.

[7] Starr, L. E., *Vet. Med.*, 1924, xix, 25.

The disease we encountered resembled in many respects the usual granular vaginitis. However, it differed from the latter in that it was often more severe, and streptococci were not found in great numbers. It will be brought out later that the streptococci we isolated are not the microbic incitant of the condition.

History of the Cases.

A considerable proportion of our cases occurred during the months of November and December, 1925, and January and February, 1926. The disease became epidemic during November shortly after a tuberculin test when a large number of newly purchased cows and a considerable number of young native cows were introduced into the herd. In several instances all the newly purchased cows and the young native cows in certain barns were attacked about the same time. The epidemic subsided but sporadic cases continued to appear in native young cows introduced into the herd during the first 5 months of 1926. During this outbreak over 100 cases occurred.

In addition to this material we had access to several cases in cows originating in Ohio and purchased from a dealer, also to cases evidently originating in Oregon among cows shipped from there to New Jersey in special cars. This material convinces us that the infection with which we had to deal is one of considerable distribution in this country.

Characterization of the Disease.

The disease was severe among the newly purchased and young native cows. The vulva was greatly swollen and tender. The visible vaginal mucosa was deeply congested and swollen and the clitoris enlarged and bright red. The mucosa covering the floor and walls of the vagina was sprinkled with numerous, tiny, indistinct, grayish white areas which rapidly coalesced to form large plaques of grayish or yellowish white exudate (Fig. 1). When the exudate was forcibly removed a raw, bleeding, grayish red surface was exposed. Considerable thick mucopurulent exudate often gathered about the clitoris and on the floor of the vagina. The inflammation slowly subsided and the exudate sloughed exposing a granulating surface. The mucosa regenerated but tiny, round, red areas appeared embedded in

the mucous membrane. These enlarged and finally became round, raised red nodules 1 to 2 mm. in diameter. A little mucopurulent exudate frequently persisted about the clitoris for a considerable period.

The lesions in the cows originating in Ohio and Oregon were much less severe. In both groups the vaginal mucosa of a number of animals was sprinkled with the red granules similar to those observed in the severe cases. In other instances a more acute condition was observed, and here the vulva was swollen and tender. The vaginal mucosa was bright red and sprinkled with strings of loosely adherent, yellowish white, purulent exudate. At times small amounts of mucopurulent exudate accumulated on the floor of the vagina. With the subsidence of the acute inflammation the characteristic granules commenced to appear.

As far as we could determine the disease was confined to the vagina. The general health was not noticeably affected. The milk yield remained normal.

Bacteriological Findings.

We attempted to demonstrate the presence of organisms in films of the exudate from fresh cases by means of heat fixation and staining with methylene blue or Gram's method. By such procedures we were able to recognize a relatively few organisms, usually streptococci or micrococci, but in insufficient numbers to account for the lesions. When rapidly dried films were fixed for 3 to 5 minutes in methyl alcohol and then stained for 30 to 40 minutes with a solution consisting of Giemsa's stain 2.0 cc., methyl alcohol 1.5 cc., distilled water 20 cc., or stained with carbolfuchsin diluted 1:20 in distilled water for 1 to 2 hours, we were able to demonstrate a considerable number of tiny delicate rods with well developed polar granules (Fig. 2). In many instances the cytoplasm between the granules stained feebly or not at all, so that the organisms resembled tiny diplococci, shown in Fig. 3.

The exudate is composed largely of leucocytes, epithelial cells, a few endothelial phagocytes, and considerable mucus. It was possible by obtaining portions of the exudate on sterile swabs and bringing the material to the laboratory to cultivate on the ordinary media

certain well defined types of organisms, such as streptococci, staphylococci, *B. coli*, and long, slender, Gram-positive rods, but in no instance was an organism encountered which resembled the bacillus met with in the films. After a considerable number of failures we were successful in obtaining it in pure culture. The procedure finally adopted was to transfer the exudate directly from the cow into the condensation water of a blood agar slant. Agar slants were prepared from veal infusion, and when slanted and cooled, 0.5 cc. of defibrinated horse blood was added. From the first tube, three others were inoculated in series, care being taken to flame the loop between each tube. The tubes were then sealed with sealing wax and incubated for 5 days at 38°C. As a rule Tube 1 contained streptococci and other types of organisms. Tube 2 contained in addition to streptococci clumps of tiny coccoids and a few tiny bacilli with well defined polar granules. Tube 3 was often to outward appearances sterile, or showed an indistinct haze in a narrow zone about the level of the condensation liquid between the tube wall and the agar; examination of the stained films, however, revealed small numbers of tiny coccoids in clumps and occasional well defined rods (Fig. 4). Tube 4 contained a pure culture or remained sterile. Transfers from the tubes containing only the coccoids and rods are usually successful, but the organism is pretty apt to grow only in the condensation fluid or between the agar and glass for four or five generations; after this time delicate flattened colonies with slightly raised centers appear on the slant.

The organism stains poorly after heat fixation, but films fixed in methyl alcohol stain well with Giemsa. It is Gram-negative and non-motile. The morphology varies. A constant finding in cultures is the densely packed masses of tiny coccoids (Figs. 4 and 6) or tiny rods (Figs. 4, 5, 6) containing polar granules. Free forms more or less elongated with well defined granules are likewise present. In later cultures (Fig. 5), the bacilli are larger and stain more deeply. All cultures passed through a phase in which growth was apparently going on more rapidly, but a final adaptation to the medium had not been reached. Here large clumps of the coccoids are plentiful, as well as extremely long filamentous forms containing large masses of deeply stained protoplasm and the tiny granules (Fig. 6). This

phase passes and finally there is a reversion to the clumps of coccoids and the tiny rods with polar granules. Cultures in blood broth reveal the general variations as illustrated in Fig. 7. It is to be observed that considerable variation in size exists. In the films of exudate the bacilli measure from 1 to 2μ in length. Bacilli of this length are common in all the cultures. The coccoids are exceedingly small, $1/3\mu$, but the probabilities are that they comprise the polar granules of bacilli whose central zones and cell walls fail to stain. The filamentous forms referred to vary from 10 to 45μ in length. Many show a tendency to fragment near the ends. Others may stain irregularly throughout their entire length.

Once a culture is established on blood agar it is readily transferred to blood broth. Thus far it has not been possible to establish growth in coagulated horse serum to which sterile calf serum water has been added or in serum agar. It will grow, however, in the condensation water of plain agar or ascitic fluid containing fresh tissue such as guinea pig spleen or kidney.

In blood broth to which 1 per cent of dextrose, lactose, saccharose, maltose, or mannitol was added, no fermentation was observed after 10 days incubation. Milk heavily inoculated with blood broth culture remained unchanged. Indole was not produced in sugar-free broth containing blood.

It seemed possible from the size of the coccoids that the organism might readily pass through the coarser Berkefeld filters. On four occasions we attempted filtration through candles V and N but the filtrates remained sterile. Inoculations from the filtrates after suitable incubation were also negative.

Pathogenicity of the Bacillus.

Rabbits weighing 2000 gm. withstand 2 cc. of blood broth culture injected intravenously. Guinea pigs of 300 gm. remain well when injected intraperitoneal with 0.5 cc. of culture. 1 cc. may cause death or produce a febrile reaction lasting several days. The injection of 2 cc. has always resulted fatally. Death results from peritonitis in 24 hours. The bacilli are found in the exudate in enormous numbers and can be cultivated from the heart's blood.

Heifer calves, 3 or 4 months old, and 2 year old heifers were in-

oculated into the vagina with culture. In every instance acute inflammation resulted. Granules similar to those observed in the spontaneous disease were always observed after the acute inflammation had subsided. The following experiment affords an example.

The mucosa of the vagina of unbred Heifer 1116 was brushed with a swab immersed in the condensation water of a 3 day blood agar culture of the bacillus in the third culture generation. There was no reaction during the first 24 hours. On the 2nd day the vulva was swollen and tender. The vaginal mucosa was bright red and swollen. Strings of yellowish white, purulent exudate adhered to the mucosa covering the floor and sides. On the 3rd day the swelling was more marked and there was considerable tenderness on manipulation. The whole mucosa was bright red and bled when brushed lightly with a sterile swab. Blood agar inoculated with exudate on this day resulted in pure cultures. The films of the exudate (Fig. 8) showed necrotic epithelial cells, leucocytes, mucus, and a moderate number of the characteristic bacilli. On the 4th, 5th, and 6th days the congestion and swelling were pronounced, and considerable exudate was present about the clitoris and adhered to the walls. Cultures made on the 5th day contained the bacilli. On the 9th day there was more exudate and the whole mucosa appeared to be granulating. After 11 days the mucosa was studded with barely visible, indistinct, grayish white areas. These were a little larger and more red in color on the 12th day, and on the 13th day were recognizable as distinctly visible, raised, red nodules. The nodules increased in size and finally on the 19th day appeared round, sharply raised, firm, 1 to 1.5 mm. in diameter. Cultures made on this day contained the bacilli.

The heifer was slaughtered 89 days after the inoculation. The granules were still visible in the mucosa of the vestibule and walls of the vagina. They did not extend into the uterus. Examination of material fixed in Zenker's fluid and stained with methylene blue revealed that the lymph follicles in the submucosa were hyperplastic. Some follicles were discrete, others were joined by bands of round cells. Over the smaller, more discrete, round celled accumulations the epithelium was normal, but that overlying the larger follicles was heavily invaded with round cells (Fig. 9).

Mention has been made that many of the cultures from the spontaneous cases contained streptococci. These were all of the non-hemolytic or green-producing type and resembled those described by F. S. Jones[8] as the type usually found in the vagina of healthy cows. We inoculated four heifer calves with the cultures. No inflammation resulted, but on subsequent inoculation with pure cultures of

[8] Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 735.

the rods from vaginitis acute inflammation resulted followed by the formation of typical granules. In these experiments the granules appeared from 5 to 7 days after infection.

We were unable to obtain material for histological study from acute spontaneous cases. On several occasions old cows leaving the herd for various reasons were inoculated into the vagina with material from severe cases, but the animals failed to contract the disease. They had probably passed through an attack of the disease and were resistant. Calves as a rule respond moderately only to inoculation with infectious material. In certain instances such inoculations induced severer inflammation and afforded some insight into the nature of the acute process.

The mucosa of the vagina of Calf 1240, 4 weeks old, was brushed with a swab containing exudate from two spontaneous cases. The usual type of acute inflammation followed. 4 days later the calf was slaughtered. At antemortem examination the mucosa was scarlet and sprinkled with strings of tenacious, yellowish white exudate. Evidently the method of slaughter, similar to that used in abattoirs, caused a blanching of the vaginal mucosa, since it was of a pale yellowish pink color along the floor and walls. Anterior to the clitoris was a red area situated within the mucosa. Other portions of the mucosa contained a few tiny, sunken, irregular, red patches. A little mucopurulent exudate was present on the mucosa of the floor. Inoculation of blood agar with this material developed cultures of the characteristic bacilli. The uterus was normal.

Histological examination of fixed and stained material revealed well defined necrosis of portions of the epithelium. In portions most of it had apparently sloughed so that the surface was covered with a thin layer of necrotic epithelial cells, degenerated leucocytes, and a little fibrin. The submucosa was edematous and infiltrated with leucocytes and round cells. The blood vessels were moderately engorged with red cells and contained excessive numbers of leucocytes and round cells. Other portions of the epithelium were intact. Lesions were not found in sections of the uterus.

From clinical examination of cases, exudate from such cases, and the histological material, we feel that the process may in part be pieced together. The bacilli attack the mucosa in certain foci. Here necrosis of the epithelium results. A little fibrin may exude beneath the epithelium. Leucocytes in large numbers invade the mucosa. The submucosa is invaded by round cells and leucocytes. The exudate and mucosa slough, followed by regeneration accompanied by

large accumulations of round cells in follicle-like masses in the sub-mucosa. The large amount of exudate in the outbreak may be explained by a heavy infection with the bacilli so that the necrotic areas occurred close together and gave the appearance of a continuous membrane.

<div align="center">DISCUSSION.</div>

It is apparent that in the large outbreak we had to deal with a severe type of inflammation of the vagina. In certain respects the type of disease differed from that usually considered typical of granular vaginitis and that encountered in our cases drawn from other sources. From each group, however, we succeeded in isolating a similar organism. The acute inflammation in all cases terminated in the appearance of the characteristic granules in the submucosa.

In the outbreak several factors contributed to exalt the severity of the infection. The disease could easily be spread by thermometers during a tuberculin test. There had been a large number of young cows recently introduced into the herd and these animals with young native cows represented a large number of susceptible individuals. The method of spread was apparently direct from cow to cow, since in this herd animals are brushed and curried before milking. It appeared that all cows on one side of a barn were infected at about the same time. A favorable opportunity was thus created for the rapid spread of the inciting organism to a large number of relatively highly susceptible cows.

With the culture isolated from the severe cases it was not possible tó produce the severe type of disease. We simply reproduced a condition similar to that found in the cows from Oregon and Ohio. It must be remembered, however, that we used for the purposes of inoculation relatively small doses of a feebly growing culture. The experimental disease was always well pronounced, accompanied by a mucopurulent exudate, and the acute process terminated in the formation of the characteristic granules. In sharp contrast are the entirely negative results after inoculation with non-hemolytic streptococci also isolated from the vaginal exudate.

Although the question of immunity produced by an attack is not

definitely proved, yet considerable resistance seems to result. We have noted on several occasions that old cows standing between severe cases did not contract the disease although infectious material must have been frequently brushed into the vagina. In two instances cows which had been exposed during the outbreak were inoculated intravaginal with material from severe cases and failed to develop the disease.

The bacillus isolated is apparently one not described before. That its distribution is widespread is indicated by its presence in cows from Oregon, Ohio, and New Jersey. Morphologically it resembles in certain respects the bipolar group in having polar granules and giving rise to the long involution forms during certain phases. However, it differs in many respects from usual organisms of this type. It fails to ferment dextrose, or any of the carbohydrates, produces no indole, and possesses relatively no pathogenicity for rabbits. It grows only in media containing blood or bits of tissue, and then only in the parts of the tubes containing little free oxygen. It is not an anaerobe. Thus far it has failed to grow in the unsealed tube. It possesses no hemolytic or proteolytic properties. At present its identification rests largely on morphological criteria, the difficulty with which it stains, and the inability to grow in media which do not contain fresh blood or tissue.

SUMMARY.

A disease of cows characterized by swelling of the vulva, acute inflammation of the vaginal mucosa, accompanied by a more or less profuse mucopurulent exudate is described. After the acute inflammation subsides the mucosa becomes studded with tiny, round, raised, red nodules which persist 2 or 3 months or longer. The acute lesion consists in necrosis of the epithelium and accumulations of leucocytes and round cells in the edematous submucosa. The nodules in the later stages are made up of densely packed masses of lymphocytes in the submucosa which force the epithelial layer outward.

A Gram-negative bacillus with tiny polar granules was found in the exudate. It measures 1 to 2μ in length and stains with difficulty. The organism was obtained in pure culture by inoculating the exudate

into tubes of slanted agar to which defibrinated horse blood had been added. Growth occurs only in sealed tubes. The organism possesses slight pathogenicity for guinea pigs. When freshly isolated cultures were introduced into the vagina of heifers or young calves, acute inflammation resulted which terminated in the characteristic granular stage of the disease.

EXPLANATION OF PLATE 15.

FIG. 1. Natural infection. Severe inflammation of the vagina. Note patches of exudate on the mucosa. About 1/5 natural size.

FIG. 2. The bacilli along the border of heavily stained mass of exudate. Spontaneous case. Methyl alcohol fixation. Dilute carbolfuchsin stain. × 1000.

FIG. 3. Exudate from spontaneous case, showing two large leucocytes and two bacilli with polar granules. Methyl alcohol fixation. Giemsa stain. × 1000.

FIG. 4. The condensation fluid from an original blood agar culture 5 days old. Note the clump of coccoids, a single well stained bacillus, and an elongated form. A red blood cell is also present. Giemsa stain, after methyl alcohol fixation. × 1000.

FIG. 5. The same culture as Fig. 4, in the fourth generation. Condensation fluid of a 5 day blood agar culture. The bacilli are larger and stain more intensely. A red blood cell is included in the field. Giemsa stain. × 1000.

FIG. 6. The same culture as Figs. 4 and 5, in the fourteenth culture generation. Condensation fluid of a 3 day blood agar culture. Three forms are illustrated, a large clump of short coccoids, a few individual bipolar forms, and two long filaments, one of which shows a tendency to fragment. Giemsa stain. × 1000.

FIG. 7. A blood broth culture, in the third generation, 3 days old. Giemsa stain. × 1000.

FIG. 8. The bacilli in the vaginal exudate from Heifer 1116, 3 days after intravaginal inoculation with culture. Giemsa stain. × 1000.

FIG. 9. Section of the mucosa of the vagina of Heifer 1116, 89 days after inoculation with culture. Note the infiltration of round cells in the mucosa and the dense accumulation of round cells in the submucosa. Zenker's fixation. Eosin-methylene blue stain. × 66.
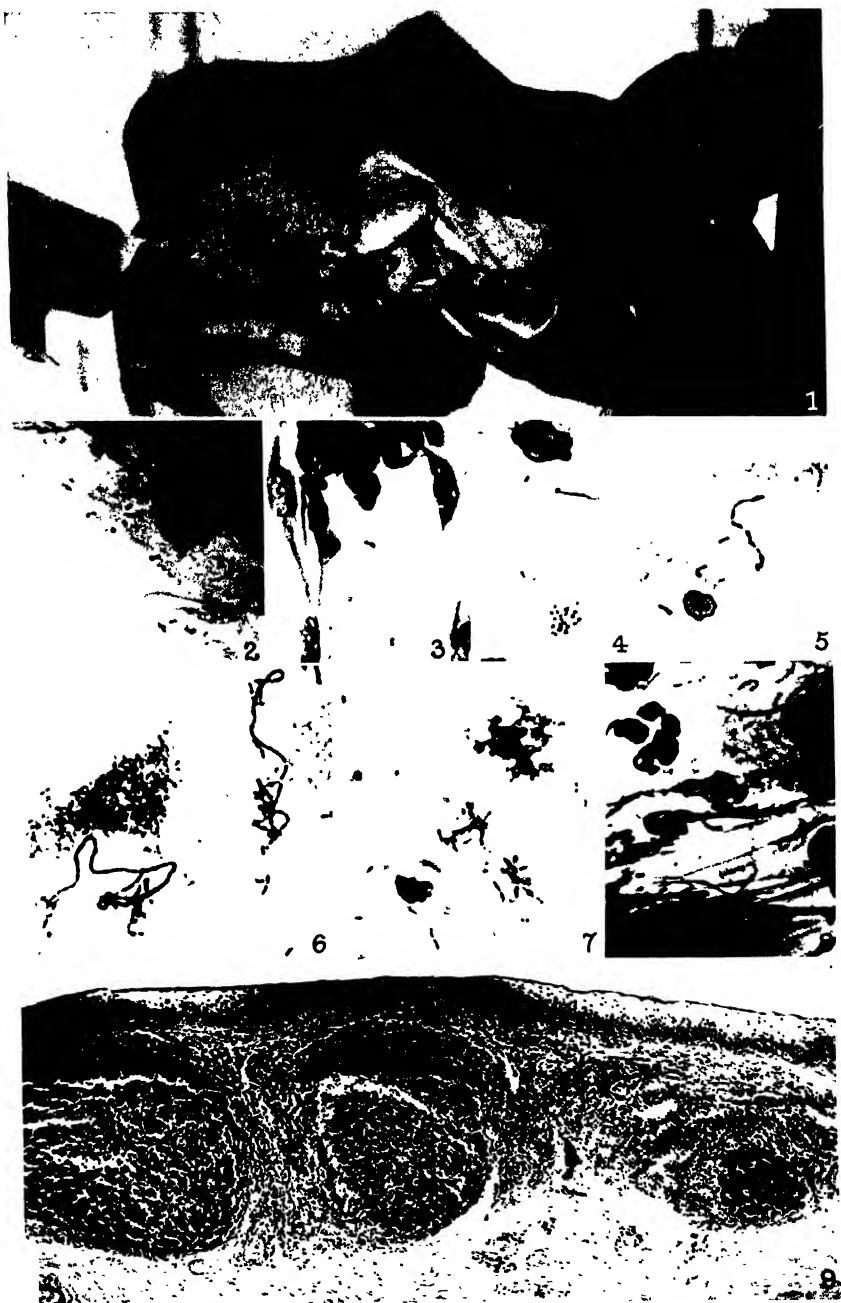
(Jones and Little: Infectious vaginitis of cows.)

STUDIES ON COMPLEMENT FIXATION IN TUBERCULOSIS. III.

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In the course of the work recorded in two previous papers (1, 2) on this subject, increasing interest has developed in a substance extractable from dried tubercle bacilli by hot alcohol, which serves as an antigen in the complement fixation reaction. Much work on the antigenic constituents of the tubercle bacillus and their chemical characterization has appeared recently. Wadsworth, Maltaner and Maltaner (3) have increased our knowledge of the lipoidal constituents and have used them in practical diagnostic work. The experience of these authors is in entire accord with my own in that, in so far as could be determined with the immune serum available to them, bacillary products when subjected to *prolonged* extraction with lipoid solvents, are deprived of residual antigenic activity. Others, particularly Dienes and Schoenheit (4, 5), while recognizing the presence and importance of the constituents extractable by the lipoid solvents, laid stress on the fact that with their sera and antigenic preparations, antigens residing with the protein fractions are also demonstrable and that some sera react with the lipoidal antigens, others with the protein. In no case, however, is it clear that their protein preparations are free from substances extractable with hot alcohol.

The work of Zinsser and Mueller (6) and of Petroff (7) emphasized the importance of "residue" after precipitation of protein-containing extracts with heat and acetic acid. These observations brought the work on the tubercle bacillus into closest conjunction with the work of Avery and Heidelberger (8) on the specifically precipitable substances derived from the pneumococcus, the active substances in the latter case being carbohydrates.

Laidlaw and Dudley (9), working with the tubercle bacillus, have separated a carbohydrate material reacting with a complement-fixing serum in dilution of 1/6,000,000 from residue remaining after prolonged contact of the bacilli with alcohol and chloroform. This substance is said to be insoluble in alcohol, but whether insoluble in hot alcohol is not stated. In many respects the substance is analogous to those obtained from the pneumococcus by Avery and Heidelberger. The possibility remains open that this substance may be found associated with either the proteins or the lipoidal fractions of the tubercle bacillus, according to the circumstances of preparation.

This possibility is emphasized by the observations of Dienes and Schoenheit (10), who have returned to the examination of the lipoidal fraction and have obtained an alcohol-soluble substance, which after a preliminary purification, reacted in the complement fixation reaction in a dilution of 1/2,000,000. The alcoholic extract in the less advanced stages of purification contained carbohydrate to the extent of 26 per cent in some instances. The carbohydrate content of the more highly purified material was not determined.

The determination of the identity or otherwise of the carbohydrates of Laidlaw and Dudley with the alcohol-soluble fraction giving carbohydrate reactions as observed by Dienes and Schoenheit, is likely also to settle finally the much debated question of whether the antigenic relationships of the tubercle bacillus to the sera of animals either naturally diseased or injected one or more times with entire tubercle bacilli, are single or multiple. Till now, it may be repeated, there is no satisfactory evidence that these more natural sera react in complement fixation with any residual material after complete extraction with hot alcohol. It is conceivable, however, that such severe extraction may injure the antigenic qualities of the residue. It is also more than probable that potentially antigenic substances may remain in completely extracted residues and will be made evident by appropriate immunological procedure even though they do not react with sera prepared with the whole bacilli.

I am now able to record a simple method for the extraction, concentration and preliminary purification of the fraction soluble in hot ethyl alcohol. The procedure we have followed should serve as a most convenient starting point for further chemical manipulation of

this portion of the lipoidal fraction. I have also found that normal serum under certain conditions gives a precipitation reaction with the material. This reaction is interesting in itself and its recognition as a possible source of confusion with true immunity reactions may be important.

<div align="center">EXPERIMENTAL.</div>

The work here recorded was all done with a rapidly growing bovine type culture of low virulence (Bovine III). Dried bacilli are extracted repeatedly in a Soxhlet extractor with absolute ethyl alcohol. When the extracts are cooled, a white precipitate forms, which is largely, although not completely, redissolved when the alcohol is again boiled. The extracts from a number of operations may be combined, boiled, filtered through paper while hot, then chilled and filtered in the ice box, giving a considerable mass of whitish paste which when quite dry can easily be ground to a powder. One or two reprecipitations under these conditions result in a powder which is completely soluble in hot alcohol to a practically colorless solution.[1] The experiments to be reported were carried out with material at this stage of purification. Weighed quantities were dissolved in hot alcohol and dilutions made from such a stock solution.

If a 0.5 to 1.0 per cent solution of this material be brought to a boil and measured with a warm pipette into 0.85 per cent NaCl solution at the temperature of the room, the 1/10 dilution is opalescent, with a bluish tinge. These solutions, or suspensions, allowed to stand for days at room or refrigerator temperature usually show no precipitation.

Such dilutions in normal saline solution freshly prepared, have been the starting point for the use of the substance in the fixation and precipitation reactions herein presented. When higher dilutions are desired, these are made by using a less concentrated alcoholic solution as a starting point, or a lesser amount of the alcoholic solution. Dilutions in series of the saline suspensions have not been practiced.

The method employed for the fixation test was fully described in our second publication. Essentially, the primary incubation is 2 hours at 37–38°, in the water bath; from 2 to 2½ units of guinea pig complement are used; approximately 10 units of hemolytic amboceptor are employed; the second incubation is ½ hour, and finally, an excess of immune serum, amounting to from 2 to 10 times the fixation unit, has been used.

When, as in this case, the antigenic value of a preparation is under consideration, the experiment may be variously interpreted. The use

[1] I am indebted to Dr. P. A. Levene for his active interest in guiding me to this simple procedure for preliminary purification of the material.

of a definite excess of complement and a large excess of hemolytic amboceptor as outlined above made our conditions very rigid. In the same sense we have considered the end-point to be at the limit of complete fixation. Dilutions of antigen giving partial fixation have been left out of account. Under these conditions the antigenic value of an ordinary sample of the material prepared as above described tested against an immune goat serum was in the neighborhood of 1/1,300,000 dilution calculated on the weight of the powder. Reading partial fixations or otherwise relaxing the conditions of the test would make it possible to recognize the presence of the antigenic substance in two- or possibly fourfold greater dilution than this, but probably a certain degree of accuracy would be lost to comparative experiments.

As an antigen this substance has advantages in the conduct of the fixation reaction. It seems to be very stable when suspended in alcohol and if these suspensions are protected from evaporation, the preparation can be regarded as a standard one, at least for a number of months. Repeated momentary re-solution by boiling, with care to limit evaporation, does not cause deterioration to an appreciable extent. The fixation unit is usually approximately 1/100 of the anti-complementary amount. 2 fixation units give a maximum effect.

Allowing for difference in technique, it would appear that this extract in hot alcohol once or twice reprecipitated by chilling out, approaches in activity the best preparations of Dienes and Schoenheit. It is also of the order of activity of the carbohydrate of Laidlaw and Dudley. This activity together with the previously determined fact that when this substance is completely removed the bacillary bodies no longer react in the complement fixation test, seem to warrant further attention to it.

In connection with fixation studies, Calmette (11) and Caulfeild (12) have repeatedly made reference to another phenomenon spoken of as the inhibition of fixation. For the demonstration as carried out by Caulfeild quantities of the specific antigen in themselves sufficient to be anticomplementary are mixed with the immune serum and incubated with the complement. If the serum is active in the inhibitory sense hemolysis will take place when the hemolytic system is completed. This is paradoxical in that either the antigen alone, or

the antigen + serum would be expected to fix or inactivate the complement.

With the antigen preparation under discussion and sera available during the past year, I made an extra effort to demonstrate the phenomenon of inhibition. The inhibition reaction has sometimes been in evidence but the experiments were marked by many irregularities and I reached no final conclusion as to their significance. The nature of the irregularities were such as to suggest that smaller serum quantities might possibly permit the demonstration where larger quantities would fail. The time factor might also be of decisive importance. I was thus led to set up unusually long dilution series and accidentally to the interesting observation that normal serum. gives a precipitation reaction with the antigen under rather unusual conditions.

The experiment fully developed after many tentative trials is as follows:

The stock alcoholic solution ($\frac{1}{2}$ per cent) of the antigen as above described, is brought to a boil and an appropriate quantity is added to 20 volumes of 0.85 per cent NaCl solution. Of this 0.9 cc. portions are put in a series of agglutination tubes. A series of serum dilutions is prepared such that when 0.1 cc. or less of each is added to the antigen in the tubes, the serum amounts will range from 0.01 cc. to 0.000002 cc. in a total volume of approximately 1 cc. The tubes are shaken and put in the ice box. The result is the formation of an abundant flocculent precipitate in a few tubes. Tubes adjacent in either direction to those showing precipitation, show an increased turbidity without the formation of flocculi. An essential condition is that the serum used shall have previously been heated to 56°C. for $\frac{1}{2}$ hour or longer. "Fresh" beef serum gives an increased turbidity without flocculation over a much wider zone. The combined results of a number of experiments with beef serum in which the above conditions were fulfilled, although at different times, are shown in Table I.

It will be noted that the precipitation centers at about concentration 0.00007, and that the turbidity change without precipitation is irregularly extended in either direction from the precipitated tubes. Serum 1116 (9-I-f) shown in Column 7 is unheated serum showing the turbidity change only.

Of the serum samples shown in this table, two numbers, 1162 and 1163, are normal, three, 1113, 1116 (13-XI) and 1118 (28-X), were from animals treated previously with avirulent bovine type bacilli,

and showing at this time moderate values in the complement fixation test. The precipitation values all approximate those of the normal animals.

TABLE I.

Serum sample*†	1163 (28-I)	1162 (28-I)	1113 (9-I)	1118 (28-X)	1116 (13-XI)	1116 (9-I-f)	1118 (26-I)
Serum amount *cc.*							
0.01	0‡	0	0	0	0	0	0
0.008	0	0	0	0	0	0	0
0.006	0	0	0	0	0	'T	0
0.004	0	0	0	0	0	T	0
0.002	0	0	0	0	0	T	0
0.001	0	0	0	0	0	T	0
0.0008	0	0	0	0	0	T	0
0.0006	0	0	T	T	0	T	0
0.0004	T	0	T	T	0	T	T
0.0002	T	+++	++++	++++	T	T	T
0.0001	++++	++++	++++	++++	++++	T	++++
0.00008	++++	++++	++++	++++	++++	T	++++
0.00006	++++	++++	++++	++++	++++	T	++++
0.00004	++++	++++	++++	++++	+++	T	++++
0.00002	++++	++++	T	T ·	T	T	++++
0.00001	0	0	T	T	T	0	0
0.000008	0	0	T	T	0	0	0
0.000006	0	0	0	T	0	0	0
0.000004	0	0	0	0	0	0	0
0.000002	0	0	0	0	0	0	0
0.000001	0	0	0	0	0	0	0

Alcoholic extract of tubercle bacilli L. 1. 0.5 per cent hot alcoholic solution diluted 1/19 with normal salt solution. Of this 0.9 cc. used in test. Serum dilutions added to total volume 1 cc. Readings after 24 hours in ice box.

* 1162 and 1163, normal calves. 1113, 1118 (28-X), 1116, calves treated with living avirulent tubercle bacilli. 1116 (9-I-f) serum not heated. 1118 (2601) calf 4 weeks after infection with virulent culture of tubercle bacillus; died 2 weeks later.

† Except 1116 (9-I-f) all sera heated to 57–58°C. for ½ hour.

‡ ++++ = complete precipitation. T = increase in turbidity without precipitation. 0 = no change in fluid.

The reaction may be developed at either 37° or at 56°C., but it is not materially hastened or more delicate at these temperatures. If the completed reaction series is placed at 56°, the flocculi contract and

the precipitate becomes heavy almost at once, but precipitation does not occur in tubes where it was not previously present. Sometimes the reaction is not fully developed in less than 48 hours.

Following Calmette's method for the separation of his inhibitory substance from the fixing substances in immune serum, serum diluted

TABLE II.

Serum	1122 (26-I)	1122 (26-I)	1122 (26-I)	1122 (9-I)	1121 (26-I)	1121 (9-I)
Preparation	Whole serum	Supernatant fluid	CO₂ precipitate*	CO₂ precipitate*	CO₂ precipitate*	CO₂ precipitate*
Serum amount cc.						
0.01	0†	0	T	T	T	T
0.008	0	0	T	T	T	T
0.006	0	0	T	T	T	T
0.004	0	0	T	T	T	T
0.002	0	0	++++	++++	++++	+++
0.001	0	0	++++	++++	++++	++++
0.0008	0	0	++++	++++	++++	++++
0.0006	T	0	++++	++++	++++	++++
0.0004	T	0	++++	++++	++++	++++
0.0002	T	T	++++	++++	++++	++++
0.0001	++++	++++	T	T	++++	++++
0.00008	++++	++++	0	T	++++	T
0.00006	++++	++++	0	0	+++	T
0.00004	++++	++++	0	0	T	T
0.00002	+++	+++	0	0	T	0
0.00001	T	T	0	0	0	0
0.000008	0	0	0	0	0	0
0.000006	0	0	0	0	0	0
0.000004	0	0	0	0	0	0
0.000002	0	0	0	0	0	0

* Serum heated to 56–57° for ½ hour before precipitation with CO_2.

† Terms as in Table I.

to 1/20 with distilled water has been precipitated with CO_2. If the CO_2 precipitate is redissolved in salt solution, and the salt content of the supernatant fluid restored, comparison shows that the CO_2 precipitate gives a similar reaction but the zone of precipitation is moved toward the stronger concentrations centering about 0.0006 instead of the tenth dilution of this amount. The fluid remaining after the

removal of the CO_2 precipitate may show no reaction whatever.   In order to achieve this complete separation it is necessary that the serum be heated to 58°C. for 2 hours before the CO_2 precipitation.   If CO_2 precipitation is done on fresh serum or on serum heated for a shorter time, or to 56°C., the separation is at least imperfect.

TABLE III.

Serum	1162	1162	1116	1116
Preparation	Supernatant fluid	CO_2 precipitate*	Supernatant fluid	CO_2 precipitate*
Serum amount				'
cc.				
0.01	0†	T	0	T
0.008	0	T	0	T
0.006	0	T	0	T
0.004	0	T	0	T
0.002	0	++++	0	++++
0.001	0	++++	0	++++
0.0008	0	++++	0	++++
0.0006	0	++++	0	++++
0.0004	0	++++	0	++++
0.0002	0	++++	0	++++
0.0001	0	++++	d.T	T
0.00008	0	T	d.T	T
0.00006	0	T	d.T	T
0.00004	0	T	d.T	0
0.00002	d.T	0	d.T	0
0.00001	0	0	d.T	0
0.000008	0	0	0	0
0.000006	0	0	0	0
0.000004	0	0	0	0
0.000002	0	0	0	0
0.000001	0	0	0	0

* Serum heated to 58–60° for 2 hours before CO_2 precipitation.
† Terms as in Table I.   d.T = questionable increase in turbidity.

Table II gives the results of serum of Calf 1122, bleeding of January 26, 1925, and compares the whole serum, the supernatant fluid after CO_2 precipitation and the redissolved CO_2 precipitate for this sample. The CO_2 precipitates of one other sample from Calf 1122 and two samples from Calf 1121 are also shown.   In this experiment the whole serum, as used in the test and also as preliminary to the CO_2 pre-

cipitation, was heated to 56–57° for ½ hour. It will be noted that the precipitations with the CO_2 precipitates are much alike. Those from Calf 1121 show a somewhat wider range. It is especially interesting that in the case of Serum 1122, the whole serum and the supernatant fluid react alike and yet the precipitate is likewise fully active.

Table III gives the results with the two samples of serum which were heated to 58–60° for 2 hours preliminary to the CO_2 precipitation. In this case the supernatant fluid is without activity while the precipitates are comparable to those shown in Table II.

The animals whose serum was used in the experiments of Table II were both infected with a virulent culture about 1 month before the samples were drawn. They had fever at this time. They had not been immunized.

The samples used in the experiments of Table III were of animals also used in those of Table I, 1162 being normal, 1116 immunized and infected.

The complement fixation reaction must, of course, be carried out with much smaller quantities of the antigen than those which show the precipitation reaction, these being anticomplementary. The fixation value of the sera in question has ranged from a maximum of 0.004 in the case of the immune sera to an immeasurable minimum of less than 0.01 in the case of the normal samples. In certain cases we have added the hemolytic system to the series after precipitation was complete, incubating the complement with the mixtures for varying lengths of time. The results have been so irregular as to be incapable of interpretation.

In one experiment, the tubes showing precipitation were pooled and filtered through paper, the filtrate being tested by complement fixation for the presence of antigen. The test was negative, showing that the specific antigenic substance with which we are dealing is included in the precipitate. The amount of serum involved in the tubes showing precipitation is much less than that required to demonstrate the fixation antibody in any of the serum samples in question and the relation of the precipitate to the immune principles of an active serum remains undetermined.

Finally the sera of a number of other species were tested; horse, sheep, goat, rabbit, guinea pig and fowl were included and all gave

precipitation. The sera were heated to 56°C. for ½ hour before the test. The reaction zones as expressed in serum concentration vary in extent and location but no distinctive character was developed by any. These results are summarized in Table IV.

TABLE IV.

Serum* of......	Horse	Sheep	Goat	Rabbit	Guinea pig	Fowl
Serum amount cc.						
0.01	0	0	0	0	0 •	T
0.008	0	0	0	0	0	T
0.006	0	0	0	0	0	T
0.004	0	0	0	0	0	T
0.002	0	0	T	0	T	++++
0.001	T	T	++++	0	T	++++
0.0008	T	++++	++++	0	T	++++
0.0006	++++	++++	++++	0	T	++++
0.0004	++++	++++	++++	T	++++	++++
0.0002	++++	++++	++++	++++	++++	++++
0.0001	++++	++++	++++	++++	++++	T
0.00008	++++	++++	++++	++++	+++	T
0.00006	T	T	++++	++++	++++	T
0.00004	T	++++	++++	++++	T	T
0.00002	T	T	T	T	T	0
0.00001	T	T	0	T	0	0
0.000008	T	0	0	T	0	0
0.000006	0	0	0	0	0	0
0.000004	0	0	0	0	0	0
0.000002	0	0	0	0	0	0

* Sera heated 56–57° for ½ hour.

DISCUSSION.

The experiments present a precipitation reaction between normal serum and an extractive preparation of the tubercle bacillus. The reaction is evidently a very delicate one considering the quantities of material involved, comparing in this respect with rather highly developed immune reactions. The quantities being adjusted on the other hand the phenomenon has the greatest constancy.

The reaction considered as an analog of immune precipitin reactions, which it certainly resembles in its main features, is **of**

interest in that it presents so wide a pro-zone. The serum dilutions in which activity is evident are quite beyond the range of those it would usually be ·thought necessary to examine in cases where the more concentrated numbers of the series failed to react, and the discovery of the precipitation was quite accidental.

The results with the fractions of serum after CO_2 precipitation, suggest some direct connection of the serum globulins with the precipitation. Conventional reasoning in consideration of the observation that in the case of serum heated to 56°C. the precipitate and the residual fluid are both active while after heating to 58°C. for a longer time, only the globulin fraction is active, would suggest that a multiplicity of substances might be involved.

The reaction may possibly be another aspect of a result obtained by Wadsworth and Maltaner (13), who found that if crude antigenic preparation from the tubercle bacillus were mixed with 5 volumes per cent of normal horse serum, and precipitated by CO_2, the antigen active in the complement fixation reaction was carried down on the globulin and could be extracted from the precipitate with alcohol.

SUMMARY.

The prolonged extraction of the tubercle bacillus with boiling ethyl alcohol, followed by one or more reprecipitations by chilling the hot alcoholic solution, easily yields a preparation very active as antigen in the complement fixation reaction.

This preparation gives a precipitation reaction with high dilution of the normal blood serum of a number of species.

The precipitation reaction presents as a peculiar feature a very long pro-zone and is further dependent on a preceding heat treatment of the serum for its demonstration.

Occurring as a reaction of normal serum, the reaction is apparently not influenced by immunization sufficient to develop moderate specific complement fixation reactions.

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